Antimutagenic Activity of Polymethoxyflavonoids from *Citrus aurantium*

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The methanol extract from *Citrus aurantium* showed a suppressive effect on *umu* gene expression of SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide). The methanol extract from *C. aurantium* was successively re-extracted with hexane, dichloromethane, butanol, and water. A dichloromethane fraction showed a suppressive effect. The suppressive compounds in the dichloromethane fraction were isolated by SiO_2 column chromatography and identified as tetra-*O*-methylscutellarein (**1**), sinensetin (**2**), and nobiletin (**3**) by EI-MS and ^1H- and ^{13}C NMR spectroscopy. These compounds suppressed the furylfuramide-induced SOS response in the *umu* test. Gene expression was suppressed 67%, 45%, and 25% at a concentration of 0.6 μmol/mL, respectively. The ID_{50} value (50% inhibition dose) of compound **1** was 0.19μmol/mL. These compounds were assayed with other mutagens, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), which requires liver metabolizing enzymes, activated Trp-P-1, and UV irradiation. These compounds showed of all mutagen-induced SOS response in the *umu* test. In addition, compounds **1**−**3** exhibited antimutagenic activity in the *S. typhimurium* TA100 Ames test.

Keywords: *Citrus aurantium; Rutaceae; polymethoxyflavonoid; umu test; SOS response; antimutagenic activity; Ames test*

INTRODUCTION

A number of *Citrus* species have been used in traditional Chinese medicine. *Citrus aurantium* (Rutaceae) is the dry unripe fruits of *C. aurantium* L., and its cultivated variants are collected when the pericarp of the fruits is still green. It is used as a digestant and expectorant and in the treatment of anal prolapse. The cultivated variants listed in the Chinese Pharmacopoeia are *C. aurantium* "Huangpi", *C. aurantium* "Daidai", *C. aurantium* "Chuluan", and *C. aurantium* "Tangcheng". Citrus species are known to produce coumarins, flavanones, flavones, flavonols, which occur in the free form and/or as glycosides, and limonoids (Benavente-Garcia et al., 1993; Bennett et al., 1991; Castillo et al., 1993).

Recently, it has been realized that a large number of natural occurring compounds have potent anticarcinogenic or antimutagenic activity against environmental carcinogen and mutagen. Mutagenic and carcinogenic agents are omnipresent in the human environment, and it seems impossible to eliminate all them. Moreover, several well-known mutagenic risk factors are closely connected with a modern lifestyle, and their entire eradication appears to be very burdensome, even unattainable. Therefore, there exists a need to reduce genotoxic effects of mutagenic and carcinogenic factors by the regular intake of antimutagenic agents. Several short-term tests for screening of environmental mutagens and carcinogens have been developed and used widely in many laboratories (Ames et al., 1975; Kada et al., 1981). The Ames test is a convenient method to evaluate mutagenic activities of these chemicals (Ames et al., 1975), 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 (McCann et al., 1975; Shugimura et al., 1976).

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). The results of this test are in agreement with the results of the Ames test and may be more useful with respect to simplicity, sensitivity, and rapidity (Reifferscheid et al., 1996).

In our search for new naturally occurring antimutagenic compounds in plants, which have a history of safe use as Chinese crude drugs (Miyazawa et al., 1997, 1998a, 1998b), we found that the methanol extract of *C. aurantium* ("Kizitu" in Japanese) exhibited a suppression of SOS-inducing activity of furylfuramide. In this paper, we report the isolation and identification of antimutagenic compounds against mutagen in *C. aurantium*.

EXPERIMENTAL PROCEDURES

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

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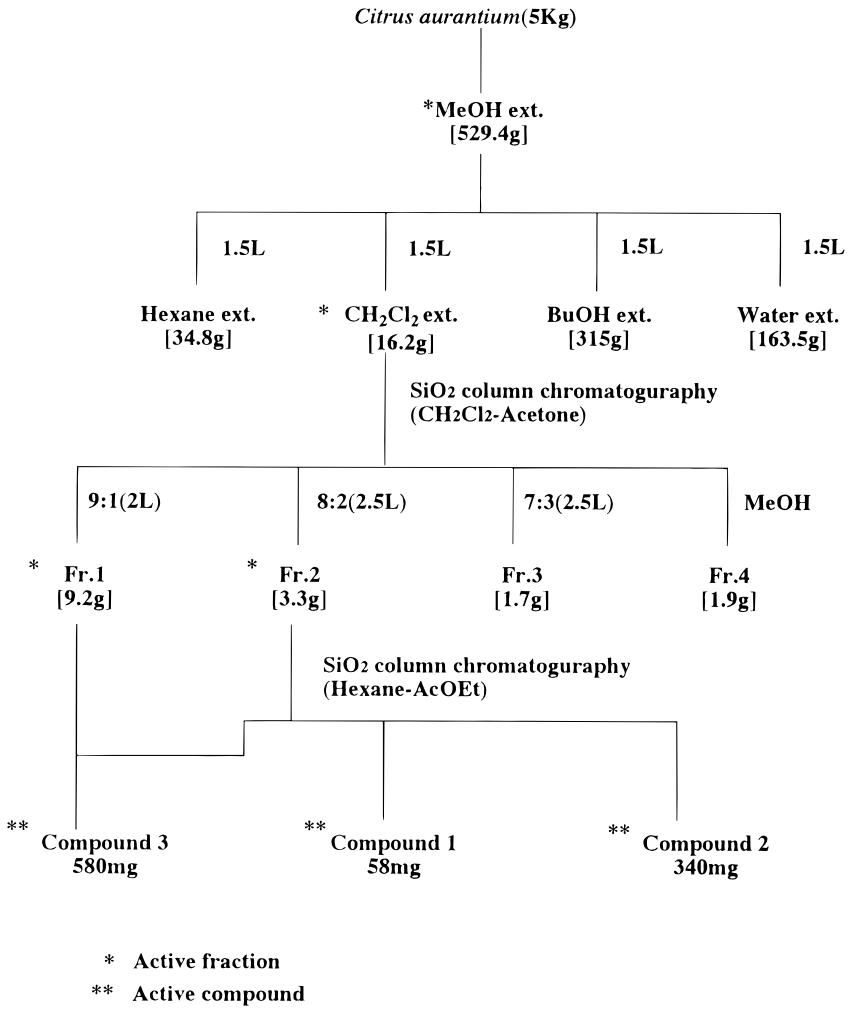


Figure 1. Isolation scheme for the suppressive compounds from *Citrus aurantium*.

on a Hewlett-Packard 5972 Series mass spectrometer interfaced with Hewlett-Packard 5890 gas chromatograph fitted with a column (HP-5MS, 30m × 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 ^{1}H 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.

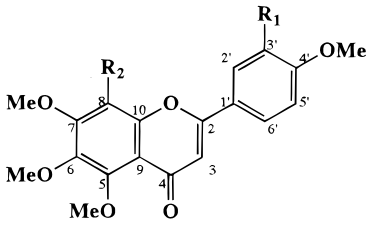
Materials. Commercially available air-dried powder of *Citrus aurantium* was obtained from Yamada Yakken Co., Ltd. (Osaka, Japan). Furylfuramide and Trp-P-1 were purchased from Wako Pure Chemical Co. (Tokyo, Japan). S9 (supernatant of 9000 g), and coenzyme, NADPH, NADH, and G-6-P were purchased from Oriental Yeast Co. (Tokyo, Japan).

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

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).

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

Fractionation and Identification of the Suppressive Compounds from *C. aurantium*. To prepare the suppressive compounds, fractionation of *C. aurantium* was carried out as described in Figure 1 using the *umu* test as a guide. The dry powder (5 kg) of *C. aurantium* was refluxed with methanol for 12 h to give a methanol extract (529.4 g). This extract was suspended in water (1.5 L) and partitioned between hexane (1.5 L) and water, dichloromethane (1.5 L) and water, and butanol (1.5 L) and water, successively. Each soluble fraction was concentrated under reduced pressure to give hexane (34.8 g), dichloromethane (16.2 g), butanol (315 g), and water (163.5 g) fractions. The dichloromethane fraction showed a suppressive effect. The fraction was fractionated to fractions 1−4 by SiO_2 column chromatography with CH_2Cl_2 and acetone as



	R_1	R_2
tetra-*O*-methylscutellarein(1)	H	H
sinensetin(2)	OMe	H
nobiletin(3)	OMe	OMe

Table 1. Suppression of Furylfuramide-Induced[a] SOS Response by Compounds 1−3 Using *S. typhimurium* TA1535/PSK1002

		dose response (μmol/mL)					
compound	furylfuramide	control[b]	0.6	0.3	0.1	0.05	ID_{50}[c]
		β-Galactosidase Activity (units)					
1	495.9	114.6	243.4	246.6	347.7	364.9	0.19
2	495.9	114.6	327.4	406.8	417.0	447.6	
3	495.9	114.6	401.5	453.6	456.8	431.8	

[a] Furylfuramide (1 μg/mL in DMSO) was added at 50 μL. [b] Control was treatment without mutagen and compounds. [c] 50% inhibition dose.

Table 2. Suppression of Trp-P-1[a] and Activated Trp-P-1[b]-Induced SOS Response by Compounds 1−3 Using *S. typhimurium* TA1535/PSK1002

				dose response (μmol/mL)					
compound	Trp-P-1	Act. Trp-P-1	control[c]	0.30	0.15	0.075	0.030	0.015	ID_{50}[d]
			β-Galactosidase Activity (units)						
1	532.3		115.2	151.9	248.3	266.1	399.2	387.0	0.024
		669.2	160.6	273.5	353.9	451.9	541.0		0.11
2	532.2		115.2	272.8	305.5	393.1	406.3	508.6	0.14
		669.2	160.6	326.3	445.1	496.7	577.1		0.19
3	532.2		115.2	308.5	333.9	388.6	413.3	438.3	0.20
		669.2	160.6	522.5	573.2	613.2	573.6		

[a] Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL. [b] Activated Trp-P-1 (10 μg/mL in DMSO) was added at 100 μL. [c] Control was treatment without mutagen and compounds. [d] 50% inhibition dose.

eluents. Fractions 1 and 2 showed suppression of SOS-inducing activity of furylfuramide in the *umu* test, and SiO_2 column chromatography and recrystallization refractionated these fractions. Finally, suppressive compounds **1** (58 mg), **2** (340 mg), and **3** (580 mg) were isolated and identified as tetra-*O*-methylscutellarein (**1**), sinensetin (**2**), and nobiletin (**3**) by GS, GS-MS, ^{1}H NMR and ^{13}C NMR spectroscopy, respectively.

Compound 1. Compound **1** was colorless needles: mp 140−142 °C; MS, *m*/*z* 342 (M^+, 15), 327 (100), 284 (10), 167 (16); IR γ_{max}, KBr (cm^{-1}) 1630, 1604, 1531, 1462, 1354, 1260; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 3.88, 3.92, 3.98, 4.00 (each 3H, s, OMe-5, 6, 7, 4′), 6.60 (1H, s, H-3), 6.82 (1H, s, H-8), 7.00 (2H, dt, J = 2, 2, 9, H-3′, 5′), 7.82 (2H, dt, J = 2, 2, 9, H-2′, 6′); ^{13}C NMR (67.8 MHz, $CDCl_3$) d 177.0 (C-4), 162.1 (C-2), 161.4 (C-4′), 157.7 (C-7), 154.4 (C-5), 152.4 (C-9), 140.3 (C-6), 127.6 (C-2′, 6′), 123.6 (C-1′), 114.3 (C-3′, 5′), 112.6 (C-10), 106.7 (C-3), 96.2 (C-8), 62.1, 61.5, 56.2, 55.4 (OMe). Compound **1** was identified as tetra-*O*-methylscutellarein [5,6,7-trimethoxy-2-(4′-methoxyphenyl)-4*H*-1-benzopyran-4-one] from these spectral data and physical properties (Chen et al., 1997).

Compound 2. Compound **2** was colorless needles: mp 178−180 °C; MS, *m*/*z* 372 (M^+, 17), 357 (100), 341 (10); IR γ_{max}, KBr (cm^{-1}) 1645, 1601, 1516, 1489, 1254; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 3.92, 3.96, 3.98 (each 3H, s, OMe), 4.00 (6H, s, 2 X OMe), 6.60 (1H, s, H-3), 6.81 (1H, s, H-8), 6.97 (2H, d, J = 9, H-5′), 7.32 (1H, d, J = 2, H-2′), 7.51 (1H, dd, J = 2, 9, H-6′); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 177.1 (C-4), 161.0 (C-2), 157.6 (C-7), 154.4 (C-5), 152.4 (C-9), 151.7 (C-4′), 149.2 (C-3′), 140.3 (C-6), 124.0 (C-1′), 119.5 (C-6′), 112.8 (C-10), 111.0 (C-5′), 108.5 (C-2′), 107.3 (C-3), 96.2 (C-8), 62.1, 61.4, 56.2, 56.0, 55.9 (OMe). Compound **2** was identified as sinensetin [2-(3′,4′-dimethoxyphenyl)-5,6,7-trimethoxy-4*H*-1-benzopyran-4-one] from these spectral data and physical properties (Chen et al., 1997; Machida et al., 1989).

Compound 3. Compound **3** was colorless needles: mp 136−138 °C; MS, *m*/*z* 402 (M^+, 22), 387 (100), 371 (6), 344 (12), 197 (20), 182 (13), 162 (8); IR γ_{max}, KBr (cm^{-1}) 1650, 1520, 1279; ^{1}H NMR (270.1 MHz, $CDCl_3$) δ 3.95(6H, s, 2 × OMe), 3.96, 3.98, 4.03, 4.11 (each 3H, s, OMe), 6.63 (1H, s, H-3), 6.99 (2H, d, J = 9, H-5′), 7.41 (1H, d, J = 2, H-2′), 7.57 (1H, dd, J = 2, 9, H-6′); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 177.3 (C-4), 160.9 (C-2), 151.9 (C-4′), 151.4 (C-7), 149.3 (C-3′), 147.7 (C-9), 144.1 (C-5), 138.0 (C-6, 8), 124.0 (C-1′), 119.6 (C-6′), 114.8 (C-10), 111.2 (C-5′), 108.6 (C-2′), 106.8 (C-3), 62.2, 61.9, 61.8, 61.6, 56.0, 55.9 (OMe). Compound **3** was identified as nobiletin [2-(3′,4′-dimethoxyphenyl)-5,6,7,8-tetramethoxy-4*H*-1-benzopyran-4-one] from these spectral data and physical properties (Chen et al., 1997; Machida et al., 1989).

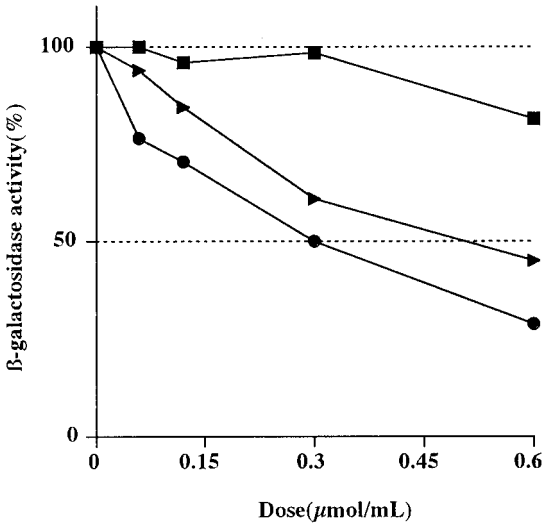


Figure 2. Suppression of UV-induced SOS responses by compound 1−3. Key: (●) effect of **1** of UV irradiation; (▲) effect of **2** of UV irradiation; (■) effect of **3** of UV irradiation. UV irradiated at 0.5 J/m^2 for 20 s.

RESULTS

Fractionation and Isolation of Suppressive Compounds from *C. aurantium*. The methanol extract of *C. aurantium* 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. The dichloromethane extract exhibited a suppressive effect on *umu* gene expression of SOS-inducing in *S. typhimurium* TA1535/pSK1002 against furylfuramide. To prepare the suppressive compounds, fractionation of dichloromethane extract was carried out as described in Figure 1. The suppressive compounds in the dichloromethane fraction were isolated by SiO_2 column chromatography and identified as tetra-*O*-methylscutellarein (**1**), sinensetin

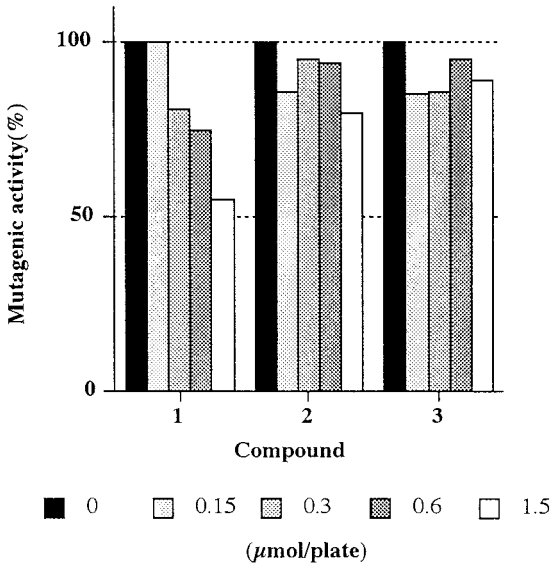


Figure 3. Effects of compounds **1**−**3** on the mutagenicity of furylfuramode in *S. typhimurium* TA100. Furylfuramide (0.5 μg/mL in DMSO) was added at 50 μL/plate.

(**2**), and nobiletin (**3**) by EI-MS and ^{1}H- and ^{13}C NMR spectroscopy.

Inhibition of Compounds 1, 2, and 3 on the SOS-Inducing Activity. The suppressive effects of compounds **1**, **2**, and **3** were determined in the *umu* test. As shown in Table 1, these compounds inhibited the furylfuramide-induced SOS response. Compounds **1**, **2**, and **3** suppressed 67%, 45%, and 25% of the SOS-inducing activity at a concentration of 0.6 μmol/mL. The ID_{50} (50% inhibition dose) value of compound **1** was 0.19 μmol/mL. These compounds were also assayed with Trp-P-1, which requires liver metabolic activation, and showed potent suppressive effects against Trp-P-1-induced SOS response (Table 2). Compounds **1**, **2**, and **3** suppressed 92%, 63%, and 54% at a concentration of 0.3 μmol/mL; ID_{50} values were 0.024, 0.14, and 0.20 μmol/mL, respectively. In addition, the suppressive effects of compounds **1**, **2**, and **3** on metabolic activation of Trp-P-1 by S9 were determined by the *umu* test. As shown in Table 2, the suppressive effects of compounds **1** and **2** on activated Trp-P-1 were greater than those of compound **3**. This result suggests that the inhibition of SOS-inducing activity of Trp-P-1, which was caused by compound **3**, is due to the inhibition of metabolic activation by S9.

Suppressive Effects of Compounds 1, 2, and 3 on UV Irradiation. Theses compounds were tested for suppressive effects on UV-induced SOS response using *Salmonella typhimurium* TA1535/pSK1002(Figure 2). Compounds **1**, **2**, and **3** suppressed the SOS-inducing activity of UV irradiation. Compounds **1** and **2** suppressed 72% and 55% of the SOS-inducing activity at a concentration of 0.6 μmol/mL, with ID_{50} values of 0.30 and 0.51 μmol/mL. On the other hand, compound **3** suppressed 20% of the SOS-inducing activity at a concentration of 0.6 μmol/mL.

Antimutagenic Activity of Compounds 1, 2, and 3. The antimutagenic activity of these compounds against furylfuramide, Trp-P-1, and activated Trp-P-1 was also demonstrated by the Ames test using *S. typhimurium* TA100. Compound **1** suppressed 45% of the mutagenicity of furylfuramide at a concentration of 1.5 μmol/plate (Figure 3). These compounds showed antimutagenic activity against Trp-P-1 (Figure 4). Compounds **1**, **2**, and **3** suppressed 70%, 75%, and 71% of the mutagenicity of Trp-P-1 at a concentration of 0.3 μmol/plate with ID_{50} values of 0.13, 0.06, and 0.06 μmol/mL, respectively. On the other hand, these antimutagenic activities against activated Trp-P-1 were remarkably decreased. Compounds **1**, **2**, and **3** inhibited 30%, 30%, and 20% of the mutagenicity of activated Trp-P-1 at a concentration of 0.30 μmol/mL. From this result, it is suggested that the antimutagenic activity of these compounds on Trp-P-1 is due to the inhibition of metabolic activation of Trp-P-1 by S9.

DISCUSSION

The antimutagenic compounds in *C. aurantium* were clearly identified as tetra-*O*-methylscutellarein (**1**), sinensetin (**2**), and nobiletin (**3**). These compounds had a suppressive effect on *umu* gene expression of SOS

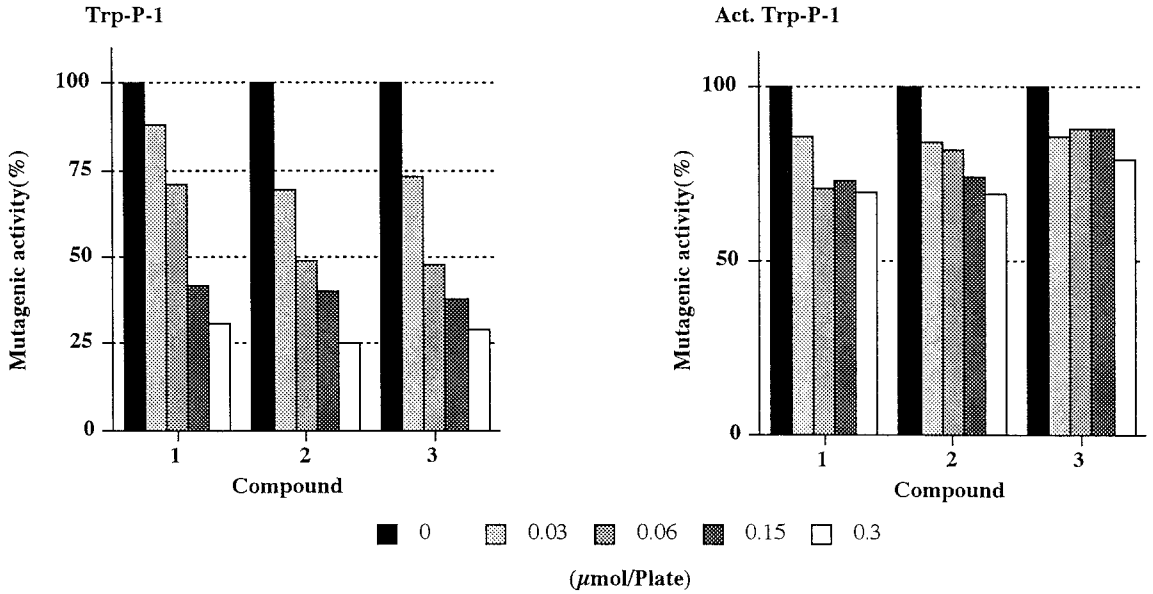


Figure 4. Effects of compounds **1**−**3** on the mutagenicity of Trp-P-1 and activated Trp-P-1 in *S. typhimurium* TA100. Trp-P-1(40 μg/mL in DMSO) was added at 50 μL/plate. Activated Trp-P-1 (10 μg/mL in DMSO) was added at 50 μL/plate.

response in *S. typhimurium* TA1535/pSK1002 against chemical mutagens, furylfuramide, and Trp-P-1. Compound **1** had the greatest suppressive potency against all chemical mutagens than that of compound **2** and **3**. The suppressive effect of these compounds is decreased with the introduction of a methoxy group at the 3′ and 8 positions. These compounds were also assayed with activated Trp-P-1 for their ability to suppress the metabolic activation of Trp-P-1 by S9 (Table 2). Compounds **1** and **2** had potent suppressive effects, but that of compound **3** is decreased compared with Trp-P-1. Compound **3** showed a suppressive effect nearly equal to that of furylfuramide. This results suggests that major inhibition of SOS-inducing activity by Trp-P-1, which was caused by compound **3**, is due to the inhibition of metabolic activation, and the characteristic activity of the respective compounds was dependent upon methyl number. As shown in Figures 3 and 4, these compounds showed antimutagenic activity against furylfuramide and Trp-P-1 using *S. typhimurium* TA100 in the Ames test while the antimutagenic activity of these compounds against activated Trp-P-1 was weaker than those of two chemical mutagens.

The antimutagenic factors are divided into the two main classes: one type is desmutagen, which inactivates or destroys mutagens directory or indirectly out of the cell, and the other type of factor is celled bioantimutagen, which suppresses the process of mutagenesis itself in the cells. These compounds inhibited the SOS-inducing activity by UV irradiation, which physical mutagen (Figure 2). These compounds inhibited SOS induction by physical mutagen. It appears that compounds **1**, **2,** and **3** might have potency for the potency of bio-antimutagen.

Flavonoids are widely distributed in the plant kingdom, and the human dietary intake of these natural products is estimated to be about 1 g/day of mixed flavonoids (Alldrick et al., 1986) and was also reported to have a wide variety physiological and biological activities, such as antioxidant action (Laughton et al., 1989), anti-scorbutic properties (Roger et al., 1988), scavenging of superoxide anions (Robaket al., 1988), modifying enzyme activity (Elliott et al., 1992). Several flavonoids have been demonstrated to have an antimutagenic effect on various mutagens or carcinogens (Francis et al., 1989; Kanazawa et al., 1995). Calomme et al.(1995) reported on inhibition of mutagenicity by *citrus* flavonoids against benzo[*a*]pyran, 2-aminofurane, quercetin, and nitroquinokine *N*-oxide. Recent research has confirmed that many higher plant contain components that possess antimutagenic and anticarcinogenic properties (Mistscher et al., 1992, 1996; Brockman et al., 1992). Wall et al. isolated various natural products, for example coumarins (1988), homoisoflavonoids (1989). In addition, the necessity for prevention of carcinogenesis has been regarded recently as being important and is expected to supply the indication on devices and improvements of ordinary diets for prevention of carcinogenesis. In this study, it is suggested that the antimutagenic compounds in *C. aurantium* were primarily methylscutellarein (**1**), sinensetin (**2**), and nobiletin (**3**). These compounds showed suppressive effects of SOS-inducing activity by chemical mutagen and UV irradiation, and the characteristic activity of the respective compounds was dependent upon methyl number.

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