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Antimutagenic Activity of Isofraxinellone from *Dictamnus dasycarpus*

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A dichloromethane extract from *Dictamnus dasycarpus* 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 suppressive compound in the dichloromethane extract from *D. dasycarpus* was isolated by SiO_2 column chromatography and identified as isofraxinellone by EI-MS and ^1H and ^{13}C NMR spectroscopy. Isofraxinellone exhibited an inhibition of the SOS-inducing activity of furylfuramide in the *umu* test. Gene expression was suppressed completely at less than 0.86 μmol/mL, and the ID_{50} value was 0.35 μmol/mL. Fraxinellone, which was one of the major components in *D. dasycarpus*, was also isolated, but this compound did not show any suppressive effect on the SOS induction of furylfuramide. Isofraxinellone 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 showed a suppressive effect similar to that with furylfuramide. Its ID_{50} value against Trp-P-1 was 0.50 μmol/mL. The antimutagenic activities of isofraxinellone against furylfuramide and Trp-P-1 were tested by an Ames test using *Salmonella typhimurium* TA100, which indicated that isofraxinellone showed antimutagenic activity.

Keywords: *Dictamnus dasycarpus; isofraxinellone; antimutagenic activity; umu test; Ames test*

INTRODUCTION

Dictamnus dasycarpus (Rutaceae) is known as a rich source of limonoids (Dreyer et al., 1972; Dreyer, 1983). Limonoids, a class of tetranortriterpenoids, constitute a group of highly oxidized natural products known to occur in the Meliaceae, Rutaceae, and Cneoraceae. Among the 37 limonoids reported to occur in citrus and its hybrids, limonin is intensely bitter and is largely responsible for delayed bitterness in citrus juices and processed products (Ozaki et al., 1991). Limonoids have also shown antifeedant activity (Serit et al., 1991; Vanucci et al., 1992; Champagne et al., 1992). Fraxinellone, isofraxinellone, and calodendrolide belong to a small group of degraded limonoids (Pailer et al., 1965; Coggon and McPhail, 1969; Fukuyama et al., 1972; Blaise and Winternitz, 1985; Boustie et al., 1990; Tokoroyama et al., 1981), and Woo et al. (1987) showed that fraxinellone has antifertility activity. Recently, the limonoid nomilin has been shown to have an effect on the inducing activity of glutathione *S*-methyltransferase (Noda, 1992). No reports on the antimutagenic activity of degraded limonoids have appeared.

In the evaluation of the carcinogenicity or mutagenicity of environmental chemicals, it is quite important to determine factors present in our environment that may affect these activities. 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. Kada et al. (1981) have studied the antimutagenic activity of foodstuffs using microbial mutation assay systems. The *umu* test system is a developed method 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).

Mutagenic and antimutagenic compounds have been found in several crude drugs, and some of these structures have been elucidated (Mizuta and Kanamari, 1985; Amonkar et al., 1986; Kim et al., 1991; Zheng et al., 1992). In our search for new naturally occurring antimutagenic compounds using plants which have a history of safe use as Chinese crude drugs (Miyazawa et al., 1995), we found that the dichloromethane extract of *D. dasycarpus* (Hakusenpi in Japanese) exhibited a suppression of the SOS-inducing activity of furylfuramide. In this paper, we report the isolation and identification of the antimutagenic compound contained in *D. dasycarpus*.

MATERIALS AND METHODS

General Procedure. Electron impact mass spectra (EI-MS) were obtained on a JEOL JMS-HX100 mass spectrometer. IR spectra were determined with a Perkin-Elmer 1760-x infrared Fourier transform spectrometer with an ordinated scale for the region 4000–450 cm^{-1}. 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

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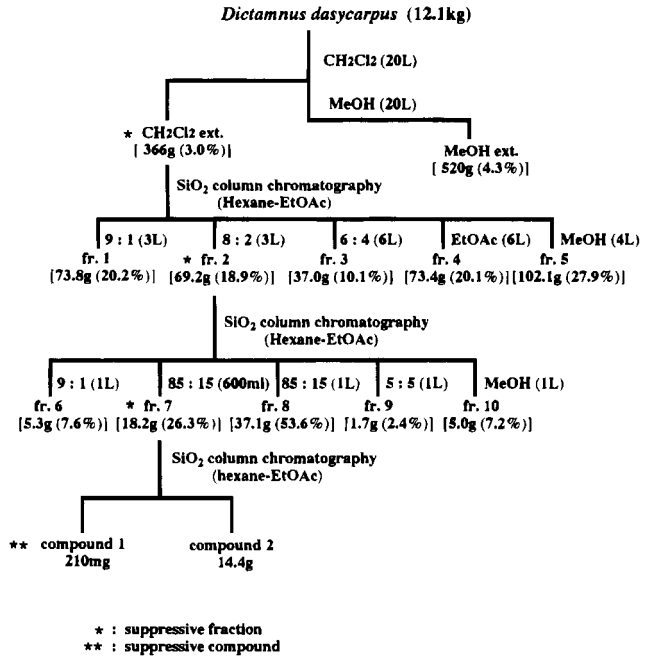


Figure 1. Isolation scheme for the suppressive compound from *D. dasycarpus*.

spectra. Specific rotation was determined with a JASCO DIP-140 digital polarimeter.

Materials. A commercially available air-dried powder of *D. dasycarpus* (Hakusenpi) was obtained from Takasago Yakugiyo Co. (Osaka). Furylfuramide and Trp-P-1 were purchased from Wako Pure Chemical Co. S9 metabolizing enzyme mixture (S9 mix) was purchased from Oriental Yeast Co.

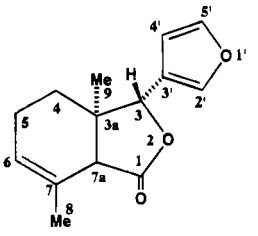
***Umu* Test.** The *umu* test for detecting the SOS-inducing activity of chemicals was carried out essentially as described by Oda et al. (1985), using *S. typhimurium* TA1535/pSK1002, plasmid pSK1002 of which carries a *umuC'-lacZ'* fused gene. SOS-inducing potency is estimated by the mesurement of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

Ames Test. The mutation test was carried out according to the preincubation method (Yahagi et al., 1977), which is a modification of Ames's method (Ames et al., 1975).

Purification and Identification of the Suppressive Compound 1. The dry powder (12 kg) of *D. dasycarpus* was refluxed with dichloromethane for 12 h to give the dichloromethane extract (Figure 1). This extract (366 g) was fractionated to fractions 1–5 by SiO_2 column chromatography (13 × 50 cm and 3 kg of silica gel) using hexane–EtOAc as eluent. The elution solvent for each fraction and fraction volumes are given in Figure 1. To pursue the compound responsible for the suppression of SOS-inducing activity, fractions 1–5 were tested. The result indicated that fraction 2 had positive activity, whereas fractions 1 and 3–5 did not show the activity. Therefore, fraction 2 was fractionated to fractions 6–10 by SiO_2 column chromatography (8 × 30 cm and 300 g), and then fraction 7 was repeatedly fractionated by SiO_2 column chromatography. Finally, suppressive compound **1** (210 mg) was isolated.

Suppressive Compound 1. Compound **1** was an oil: $[\alpha]^{20}_D$ +14.7° ($CHCl_3$; *c* 0.85); high-resolution EI-MS, calcd for $C_{14}H_{16}O_3$ (M^+) 232.1100, obsd 232.1075; IR γ^{KBr}_{max} cm^{-1} 1767 (γ-lactone), 3144, 1504, 1029, 876, 805 (furan); ^1H NMR, ^{13}C NMR, and EIMS identical with those of isofraxinellone (Blaise and Winternitz, 1985). The suppressive compound **1** was identified as isofraxinellone [3α-(3′-furanyl)-3a,7-dimethyl-3a,4,5,7a-tetrahydroisobenzofuranone] from these spectral data and physical properties.

Purification and Identification of the Isomer (Compound 2) of Suppressive Compound 1. For the purposes of comparison with **1**, compound **2** (14.4 g), which is an isomer of **1**, was also isolated from fraction 7 (Figure 1).



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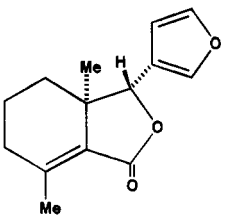

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Table 1. Suppressive Effect of *D. dasycarpus* Fractions on Furylfuramide[a] Using *S. typhimurium* TA1535/pSK1002

		dose–response[c]			
sample	control[b]	200	100	50	0 (μg/mL)
CH_2Cl_2 ext	240.3	570.0	635.2	716.8	921.1
MeOH ext	240.3	881.1	890.5	902.3	921.1
fraction 1	249.9	680.3	820.3	873.6	890.5
fraction 2	249.9	377.9	574.5	734.6	890.5
fraction 3	249.9	657.7	837.2	905.2	890.5
fraction 4	249.9	770.9	806.2	909.6	890.5
fraction 5	249.9	850.2	898.5	860.3	890.5
fraction 6	262.9	754.4	905.4	992.2	1044.4
fraction 7	262.9	303.5	544.2	812.2	1044.4
fraction 8	262.9	670.9	719.3	892.5	1044.4
fraction 9	262.9	760.5	822.3	968.9	1044.4
fraction 10	262.9	829.3	878.5	1032.9	1044.4

[a] Furylfuramide (1 μg/mL in DMSO) was added at 60 μL. [b] Control was a treatment without furylfuramide. [c] β-Galactosidase activity (units).

Compound 2 (Isomer of Suppressive Compound 1). Compound **2** was needles: mp 114–115 °C; $[\alpha]^{20}_D$ −38.3° ($CHCl_3$; *c* 1.00); high-resolution EI-MS, calcd for $C_{14}H_{16}O_3$ (M^+) 232.100, obsd 232.1074; IR γ^{KBr}_{max} cm^{-1} 1750 (γ-lactone), 1674 (conjugated C=C–C=O), 3123, 1600, 1502, 1030, 875, 802 (furan); ^1H NMR, ^{13}C NMR, and EIMS identical with those of fraxinellone (Blaise and Winternitz, 1985). Compound **2** was identified as fraxinellone [3α-(3′-furanyl)-3a,7-dimethyl-3a,4,5,6-tetrahydroisobenzo furanone) from these spectral data and physical properties.

RESULTS AND DISCUSSION

Fractionation of the Extract of *D. dasycarpus* and Isolation of Isofraxinellone (1) and Fraxinellone (2). As shown in Figure 1 and Table 1, the dichloromethane extract from *D. dasycarpus* showed a suppressive effect on *umu* gene expression of the SOS responses in *S. typhimurium* TA1535/pSK1002 against furylfuramide. The methanol extract did not show this suppressive effect. The dichloromethane extract was fractionated to search for the suppressive compound 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. Only fraction 2 had a clear-cut dose–response effect in the first fractionation (fractions 1–5) by SiO_2 column chromatography. Therefore, suppressive fraction 2 was fractionated to fractions 6–10 by further SiO_2 column chromatography. From the results of the *umu* test of fractions 6–10, fraction 7 had the effect of suppressing the SOS-inducing activity. The suppressive fraction 7 was fractionated by repeated SiO_2 column chromatography to yield compound **1**. Another compound (compound **2**), which was the major constituent of this fraction, was also isolated from fraction 7.

Structure Determination of Isofraxinellone (1) and Fraxinellone (2). Compound **1** gave a molecular ion as a base peak in the high-resolution EI mass

Table 2. Suppressive Effect of Isofraxinellone and Fraxinellone on Furylfuramide[a] and Trp-P-1[b] Using *S. typhimurium* TA1535/pSK1002

chemical	furylfuramide	Trp-P-1	control	dose–response[c] 0.86	0.65	0.45	0.28	0.09 (μmol/mL)
isofraxinellone	902.2		217.0	192.9	300.4	450.2	639.5	826.8
fraxinellone	902.2		217.0	790.3	835.4	895.3	905.6	900.6
isofraxinellone		405.5	123.5	110.2	203.2	281.5	341.0	385.7
fraxinellone		405.5	123.5	362.3	386.5	405.6	403.3	395.6

[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] β-Galactosidase activity (units).

spectrum at m/z 232.1075, which corresponded to the formula $C_{14}H_{16}O_3$ (calcd 232.1100). The ^1H NMR spectram of **1** confirmed the presence of a methyl group at δ 0.89 (Me-3a) and 1.93 (Me-7), and the signals at δ 6.34 (H-4′), 7.41 (H-2′), and 7.45 (H-5′) could be assigned to the furan ring. The signals at H-3, H-6, and H-7a appeared at δ 5.91, 5.65, and 2.74, respectively. Thus, compound **1** was identified as isofraxinellone. Compound **2** showed a molecular ion at m/z 232.1074 in the high-resolution EI mass spectrum, corresponding to the elemental composition $C_{14}H_{16}O_3$ (calcd 232.1100). The ^1H and ^{13}C NMR spectra of compound **2** indicated the appearance of a 7–7a double bond and the disappearance of a 6–7 double bond, which further confirmed the structure of **2** as fraxinellone.

Inhibition of the SOS-Inducing Activity of Isofraxinellone (1) and Fraxinellone (2). The suppressive effect of compounds **1** and **2** was determined in the *umu* test. As shown in Table 2, isofraxinellone exhibited inhibition of the SOS induction by furylfuramide, whereas fraxinellone did not. Isofraxinellone completely suppressed SOS induction at 0.86 μmol/mL, and the ID_{50} value was 0.35 μmol/mL. In addition, isofraxinellone and fraxinellone were assayed with another mutagen (Trp-P-1), which requires liver metabolizing enzymes for mutagenicity. In this case, isofraxinellone showed a suppressive effect similar to that with furylfuramide, whereas fraxinellone did not. The ID_{50} value of isofraxinellone against Trp-P-1 was 0.50 μmol/mL.

Antimutagenic Activity of Isofraxinellone (1) and Fraxinellone (2). The antimutagenic activity of these compounds against furylfuramide and Trp-P-1 was also demonstrated by the Ames test using *S. typhimurium* TA100. As shown in Figure 2, isofraxinellone suppressed the mutagenicity of furylfuramide completely at 0.65 μmol/mL, and the ID_{50} value was 0.24 μmol/mL. Similarly, isofraxinellone suppressed the mutagenicity of Trp-P-1 completely at 0.73 μmol/mL, and the ID_{50} value was 0.30 μmol/mL. Fraxinellone did not suppress the mutagenicity of either compound.

Thus, the antimutagenic component in *D. dasycarpus* was clearly identified as isofraxinellone, while fraxinellone had no antimutagenic activity. The difference in structure between isofraxinellone and fraxinellone is the position of the double bond in the cyclohexene ring. Isofraxinellone has a 6–7 double bond, while fraxinellone has a 7–7a double bond. Clearly, the presence of a double bond at the 6–7 position is important for antimutagenic activity.

In previous papers, Mizuta and Kanamari (1985) and Kanamori et al. (1986) reported on the mutagenic activity of dictamine and γ-fagarine from the same crude drug (*D. dasycarpus*). Our research did not confirm the SOS-inducing activity of either the dichloromethane or methanol extract from *D. dasycarpus*. For this reason, we think that the minor constituents of this crude drug may vary depending on differences in the place of production, harvest time, or method of preservation.

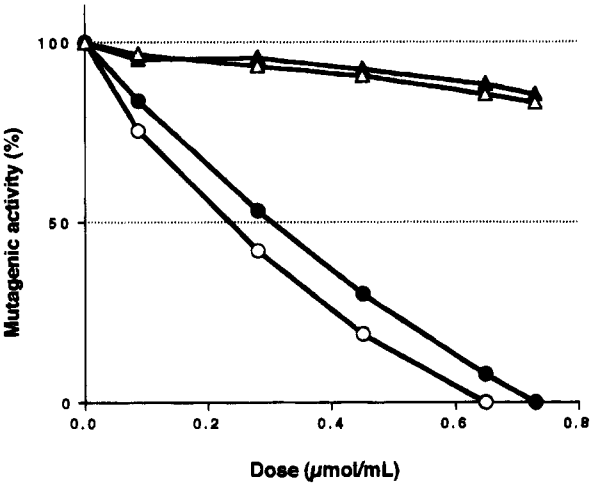

Figure 2. Effect of isofraxinellone and fraxinellone on the mutagenicity of furylfuramide and Trp-P-1 in *S. typhimurium* TA100: (○) effect of isofraxinellone on the mutagenicity of furylfuramide; (●) effect of isofraxinellone on the mutagenicity of Trp-P-1; (△) effect of fraxinellone on the mutagenicity of furylfuramide; (▲) effect of fraxinellone on the mutagenicity of Trp-P-1. Furylfuramide (1 μg/mL in DMSO) was added at 50 μL/plate. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL/plate.

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