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ARTICLE

Suppression of SOS-Inducing Activity of Chemical Mutagens by Metabolites from Microbial Transformation of (−)-Isolongifolene

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In this study, biotransformation of (−)-isolongifolene (**1**) by *Glomerella cingulata* and suppressive effect on *umuC* gene expression by chemical mutagens 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide) and aflatoxin B_1 (AFB_1) of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 were investigated. Initially, **1** was carried out the microbial transformation by *G. cingulata*. The result found that **1** was converted into (−)-isolongifolen-9-one (**2**), (−)-(2*S*)-13-hydroxy-isolongifolen-9-one (**3**), and (−)-(4*R*)-4-hydroxy-isolongifolen-9-one (**4**) by *G. cingulata*, and their conversion rates were 60, 25, and 15%, respectively. The metabolites suppressed the SOS-inducing activity of furylfuramid and AFB_1 in the *umu* test. Comound **2** showed gene expression by chemical mutagens furylfuramide and AFB_1 was suppressed 54 and 50% at <0.5 mM, respectively. Compound **2** is the most effective compound in this experiment.

KEYWORDS: SOS-inducing activity; furylfuramide; AFB_1; *umu* test; microorganism; (−)-isolongifolene; (−)-isolongifolen-9-one; (−)-(2*S*)-13-hydroxyisolongifolen-9-one; (−)-(4*R*)-4-hydroxyisolongifolen-9-one

INTRODUCTION

Microbial transformation is a biologically synthetic process, using enzymes in the living body as biocatalysts. The characteristics of biotransformation are as follows: regio- and stereoselective reaction under mild condition producing optically active compounds (*1*−*5*). Biotransformation to a new biologically active compound from terpenoids of plant ingredients makes use of plant pathogenic fungi, which is put to practical use in nature.

(−)-Isolongifolene (**1**), a sesquiterpene hydrocarbon with a longifolene skeleton, was contained in the essential oil of the cone of pine and Japanese cedar (*6*−*8*). This compound showed a suppressive effect on *umuC* gene expression in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide). Gene expression was suppressed 50% at 1.0 mM **1**.

The SOS response appears to be induced by an alteration in DNA synthesis, either directly by DNA damage blocking agents to the replication fork or indirectly by antibiotics, such as novobiocin, that inhibit DNA synthesis. The *umu* test system was developed to evaluate the genotoxic activity of a wide variety of environmental carcinogens and mutagens, using the expression of one of the SOS genes to detect DNA-damaging agents (*9*, *10*). The results of this test are in agreement with those of the Ames test, and it may be more useful with respect to simplicity, sensitivity, and rapidity.

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As a part of our continuing program to discover bioactive natural compounds, we have investigated antimutagenic compounds and suppressive compounds of SOS response in plants (*11*, *12*). However, there are few reports about the suppressive effect on chemical mutagen-induced SOS response by sesquiterpenoids. Therefore, in this study, we report the suppressive effect of sesquiterpenoids with the longifolene skeleton against chemical mutagen-induced SOS response.

MATERIALS AND METHODS

General Procedure. NMR was performed at 500 MHz (^{1}H) and 125 MHz (^{13}C), $CDCl_3$, with TMS as internal standard. For GC, a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector, a DB-5 capillary column (30 m length, 0.25 mm i.d), and a split injection of 20:1 was used. Helium at a flow rate of 1 mL/min was used as a carrier gas. The oven temperature was programmed from 90 to 270 °C at 4 °C/min. The injector and detector temperatures were 270 and 280 °C, respectively. System software control and data analysis were performed with GC Chemstation software (Agilent Technologies Inc.). For GC-MS, a Hewlett-Packard 5890A gas chromatograph equipped with a split injector HP-5MS capillary column (30 m length, 0.25 mm i.d.) was combined by direct coupling to a Hewlett-Packard 5972A mass spectrometer. The same temperature program as just described for GC was used. Helium at 1 mL/min was used as a carrier gas. The temperature of the ion source was 280 °C, and the electron energy was 70 eV. The electron impact (EI) mode was used. IR spectra were obtained with a Perkin-Elmer 1760X spectrometer; $CHCl_3$ was used as a solvent. TLC used silica gel 60 F254 precoated (layer thickness = 0.25 mm, Merck), and CC used silica gel developed with a hexane−EtOAc gradient.

Materials. The substrate used for the biotransformation experiments was (−)-isolongifolene (Fluka). Furylfuramide and aflatoxin B_1 (AFB_1) were purchased from Wako Pure Chemical Co., Osaka, Japan.

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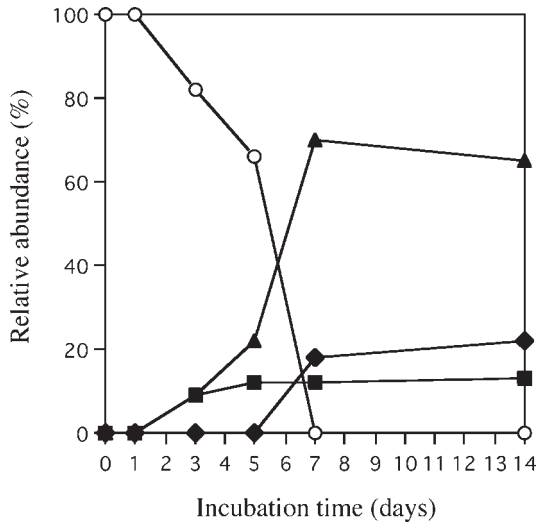

Figure 1. Time course of the biotransformation of **1** by *G. cingulata*: ○, (−)-isolongifolene (**1**); ■, (−)-isolongifolen-9-one (**2**); ▲, (−)-(2*S*)-13-hydroxy-isolongifolen-9-one (**3**); ◆, (−)-(4*R*)-4-hydroxy-isolongifolen-9-one (**4**).

S9 metabolizing enzyme mixture (S9-mix) was purchased from Oriental Yeast Co., Tokyo, Japan.

Preculture of *Glomerella cingulata*. Spores of *G. cingulata*, which had been preserved at low temperature, were inoculated into sterilized culture medium (1.5% saccharose, 1.5% glucose, 0.5% polypeptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.1% K_2HPO_4, 0.001% $FeSO_4 \cdot 7H_2O$, and distilled water, pH 7.2) in a shaking flask at 27 °C for 5 days.

***Umu* Test.** The *umu* test for detecting the SOS-inducing activity of chemicals was carried out according to the method of Miyazawa et al. (*4*) using *S. typhimurium* TA1535/pSK1002, the plasmid pSK1002 of which carries an *umuC′-′lacZ* fused gene.

Briefly, the overnight culture of tester bacteria strain (*S. typhimurium* TA1535/pSK1002) in Luria broth (1% bactotryptone, 0.5% NaCl, and 0.5% yeast extract; supplemented with 50 μg/mL ampicillin) was diluted 50-fold with fresh TGA medium (1% bactotryptone, 0.5% NaCl, and 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 bacterial culture was subdivided into 2.1 mL portions in test tubes, and test compound (50 μL, in DMSO), 0.1 m phosphate buffer (300 μL, pH7.4), and mutagens, which was furylfuramide or AFB_1 (50 μL, in DMSO), were added to each tube. In the case of AFB_1, S9 mix was added in each tube instead of phosphate buffer. As positive control, an equivalent volume of DMSO was added instead of test compound, whereas with negative controls, an equivalent volume of DMSO was added instead of both test compound and mutagens. After 2 h of incubation at 37 °C with shaking, the culture was centrifuged to collect cells, which were resuspended in 2.5 mL of PBS. The optical densities of the suspensions at 600 nm were recorded with one portion (0.25 mL), whereas the rest of the cell suspensions was used to measure the level of intracellular β-galactosidase activity using the method of Miller (1972).

Time Course Experiment *G. cingulata*. Precultured *G. cingulata* was transferred into a 300 mL Erlenmeyer flask containing 200 mL of culture medium and stirred for 3 days. After the growth of *G. cingulata*, **1** (60 mg) was added to the medium and the culture continued for 15 days; then the medium was salted out with NaCl and extracted with EtOAc. The extract was analyzed by GC. The ratios between the substrate **1** and metabolic products were determined on the basis of the peak areas of GC and GC-MS (**Figure 1**).

Isolation of Metabolites. Precultured *G. cingulata* was transferred into 2 L stirred incubation. Cultivation was carried out at 27 °C with stirring for 3 days under aeration. After the growth of *G. cingulata*, **1** (600 mg) was added into the culture medium and the culture continued for 7 days.

After the biotransformation for 7 days, culture medium and mycelia were separated by filtration. The medium was salted out with NaCl and extracted with EtOAc. The mycelia were also extracted with EtOAc. The EtOAc extracts were mixed and dried over Na_2SO_4, and the solvent was evaporated to yield the crude extract (784 mg). The extract was chromatographed on silica 300 columns with hexane−EtOAc; metabolites **2** (55 mg), **3** (350 mg), and **4** (121 mg) were isolated.

Table 1. ^{1}H NMR Spectral Data of Compounds **2**−**4** (δ, TMS, in $CDCl_3$, at 500 MHz)[a]

	2	3	4
3	1.94 (1H, *t*, 3.4)	2.19−2.25 (1H, *m*)	1.96−1.98 (1H, *m*)
4	1.90 (1H, *dd*, 3.9, 12.1)	1.65−1.76 (2H, *m*)	4.33−4.35 (1H, *m*)
5	1.37 (1H, *m*)	1.60−1.65 (2H, *m*)	1.75 (1H, *ddd*, 1.1, 2.1, 11.9)
	1.57 (1H, *m*)		1.83 (1H, *ddd*, 2.6, 6.8, 13.0)
8	2.05 (1H, *m*)	2.04−2.07 (1H, *m*)	2.06−2.10 (1H, *m*)
	2.35 (1H, *m*)	2.35−2.45 (1H, *m*)	2.32−2.35 (1H, *m*)
10	5.67 (1H, *s*)	5.67 (1H, s)	5.72 (1H, *s*)
11	1.47 (1H, *m*)	1.98 (2H, *td*, 8.4, 13.5)	1.60−1.63 (1H, *m*)
	1.66 (1H, *m*)		1.88−1.91 (1H, *m*)
12	1.11 (3H, *s*)	1.00 (3H, *s*)	1.12 (3H, *s*)
13	1.05 (3H, *s*)	3.62 (1H, *d*, 10.8)	1.13 (3H, *s*)
		3.53 (1H, *d*, 10.8)	
14	1.01 (3H, *s*)	1.19 (3H, *s*)	1.00 (3H, *s*)
15	0.96 (3H, *s*)	0.96 (3H, *s*)	1.08 (3H, *s*)

[a] Coupling constasnts in hertz.

Table 2. ^{13}C NMR Spectral Data for Compounds **2**−**4** (δ, TMS, in $CDCl_3$ at 125 MHz)[a]

	2	3	4
1	187.6	179.6	181.5
2	45.3	49.3	42.0
3	47.9	43.1	54.4
4	25.2	24.2	70.3
5	37.6	36.5	40.2
6	60.2	58.5	57.9
7	35.5	34.2	34.1
8	50.7	49.9	49.3
9	202.9	199.9	199.7
10	117.3	117.9	117.6
11	28.9	34.2	32.8
12	27.2	21.9	24.2
13	25.6	67.3	27.1
14	25.9	25.9	25.2
15	24.8	25.3	25.9

[a] Chemical shifts in parts per million; multiplicities were determined by the DEPT pulse sequence.

(−)-*Isolongifolen-9-one* (*2*). **2** as obtained as a colorless crystal: $[\alpha]^{21.5}_D$ −202° (*c* 1.0, CH_3OH); EIMS *m*/*z* (relative intensity) $[M]^+$ 218 (53), 203 (15), 189 (13), 175 (100), 162 (72), 147 (61), 91 (35), 41 (28); IR (KBr) $\nu_{max}cm^{-1}$ 2965, 1650; ^{1}H and ^{13}C NMR data shown in **Tables 1** and **2**.

(−)-(*2S*)-*13-Hydroxy-isolongifolen-9-one* (*3*). **3** was obtained as a colorless oil: $[\alpha]^{21.5}_D$ −162° (*c* 1.0, $CHCl_3$); EIMS *m*/*z* (relative intensity) $[M]^+$ 234 (53), 203 (55), 176 (100), 147 (14), 119 (17), 91 (66), 55 (33); IR (KBr) $\nu_{max}cm^{-1}$ 3429, 2965, 1653; ^{1}H and ^{13}C NMR data as shown in **Tables 1** and **2**.

(−)-(*4R*)-*4-Hydroxy-isolongifolen-9-one* (*4*). **4** was obtained as a colorless oil: $[\alpha]^{19.7}_D$ −74.6° ($CHCl_3$; *c* 1.0); EIMS *m*/*z* (relative intensity) $[M]^+$ 234 (63), 216 (16), 178 (58), 160 (51), 150 (59), 135 (80), 85 (100), 41 (90); IR (KBr) ν_{max} cm^{-1} 3420, 2963, 1652; ^{1}H and ^{13}C NMR data as shown in **Tables 1** and **2**.

RESULTS AND DISCUSSION

Biotransformation of (−)-Isolongifolene (1) by *G. cingulata*. Biotransformation of (−)-isolongifolene (**1**) by *G. cingulata* was investigated. For time course experiments, a small amount of **1** was incubated with *G. cingulata* for 14 days. Compound **1**

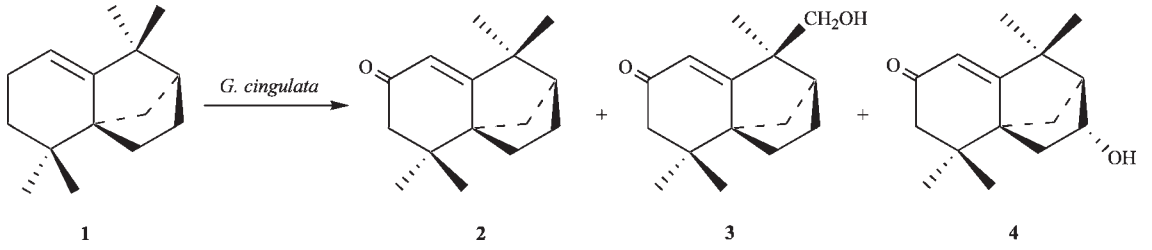

Figure 2. Biotransformation of (−)-isolongifolene by *G. cingulata*.

was transformed to three metabolites by *G. cingulata*, which were detected by TLC, GC, and GC-MS analyses. These products were not detected on TLC, GC, and GC-MS analyses of a culture of microorganism to which no substrate was fed. **Figure 1** shows the time course for the appearance of the metabolites.

To isolate the metabolites, a large-scale incubation of **1** with fungi was carried out, and the culture was extracted as described under Materials and Methods. These metabolites were isolated from the EtOAc extract, and the structure was determined by spectral data.

Compounds **2** and **3** were determined to be (−)-isolongifolen-9-one (**2**) and (−)-(2*S*)-13-hydroxy-isolongifolen-9-one (**3**) as compared to past reports from the following MS, IR, and NMR data, respectively (*13*−*16*).

Metabolite **4**, IR, and ^{13}C NMR spectrum of **4** showed the presence of a hydroxyl group (IR, 3420 cm^{-1}; δ_C 70.3 (CH)) and a carbonyl group (IR, 1652 cm^{-1}; δ_C 199.7 (C)). ^{1}H and ^{13}C NMR signals were similar to those of the substrate, except for the existence of a new methine group and the disappearance of a methylene group. The ^{1}H NMR evidenced the presence of a three-methyl group. This was confirmed by assignment of the NMR spectra using two-dimensional techniques (correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple-bond connectivity (HMBC)). In the characteristic HMBC, spectrum correlation was observed of two nonproton carbons (δ_C 42.0 and 57.9; C-2 and C-6) with a new methine proton (δ_H 4.33−4.35; C−H). Therefore, compound **4** was produced by hydroxylation at the C-4 position of **1**. The configuration of C-4 as *R* was inferred from NOE experiment: when the signal of compound **4** of Me-13 was irradiated, an enhancement of the H-4 was observed. However, NOE correlations between Me-12 and H-4 were not observed, indicating the α-position of the hydroxyl group at C-4. The specific rotation shows the (−)-form. From these data it was concluded that the structure of **4** is (−)-(4*R*)-4-hydroxy-isolongifolen-9-one (**4**). Therefore, compound **1** was biotransformed to (−)-isolongifolen-9-one (**2**), (−)-(2*S*)-13-hydroxy-isolongifolen-9-one (**3**), and (−)-(4*R*)-4-hydroxy-isolongifolen-9-one (**4**) by *G. cingulata* (**Figure 2**).

Suppressive Effect on *UmuC* Gene Expression by Chemical Mutagens Furylfuramide and AFB$_1$. The suppressive effects of compounds **1**−**4** were determined in the *umu* test, and cell growth was unaffected at a concentration of these compounds under experimental condition. As shown in **Figure 3**, **1** showed a suppressive effect on *umu* gene expression of the SOS responses in *S. typhimurium* TA1535/pSK1002 against furylfuramide. Compound **1** suppressed 56% of the SOS-inducing activity at concentrations of < 1.0 mM, and the IC_{50} value was 0.77 mM. Compound **2** suppressed 54% of the SOS-inducing activity at concentrations of < 0.5 mM, although this compound showed toxicity at 1.0 mM. Compounds **3** and **4** suppressed 38.8 and 40.2% of the SOS-inducing activity at concentrations of < 1.0 mM, respectively. **Figure 4** shows suppressive effects on

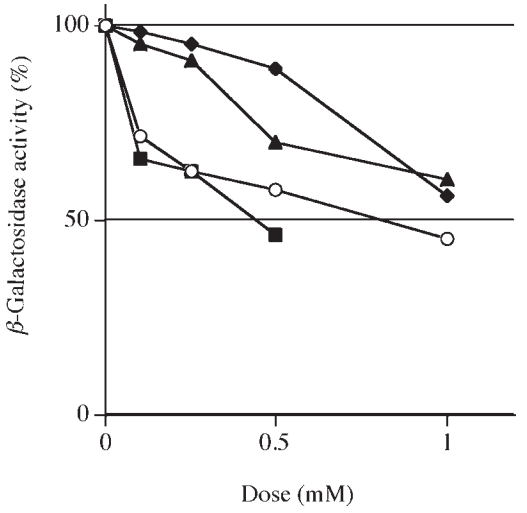

Figure 3. Suppression of furylfuramide-induced SOS responses by compounds **1**−**4**: ○, effect of **1**; ■, effect of **2**; ▲, effect of **3**; ◆, effect of **4**. Furylfuramide (3.3 μg/mL in DMSO) was added at 10 μL. Data represent the mean from four experiments.

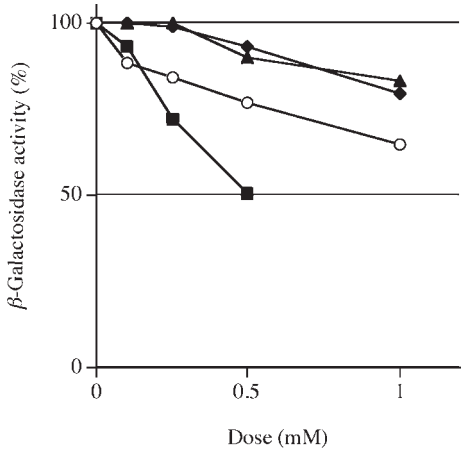

Figure 4. Suppression of AFB$_1$-induced SOS responses by compounds **1**−**4**: ○, effect of **1**; ■, effect of **2**; ▲, effect of **3**; ◆, effect of **4**. AFB$_1$ (33 μg/mL in DMSO) was added at 10 μL. Data represent the mean from four experiments.

AFB$_1$-induced SOS response. Compounds **1**, **3**, and **4** showed suppressive effects of SOS-inducing activity, and these compounds showed 35, 19, and 21% suppressive effects at concentration of < 1.0 mM, respectively. Compound **2** suppressed 50% of the SOS-inducing activity at concentrations of < 0.5 mM, and **2** is the most effective compound in this experiment. Compound **2** was shown to have anti-SOS-inducing activity against AFB$_1$. We suggest that **2** could specifically inhibit cytochrome P450 3A4 (CYP3A4), because AFB$_1$ is catalyzed by CYP3A4.

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