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Suppression of SOS-Inducing Activity of Chemical Mutagens by Metabolites from Microbial Transformation of (+)-Longicyclene

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In this study, biotransformation of (+)-longicyclene (**1**) by *Aspergillus niger* (NBRC 4414) and the suppressive effect on *umuC* gene expression by chemical mutagens 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (furylfuramide) and aflatoxin B_1 (AFB1) of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 were investigated. Initially, compound **1** was converted to three new terpenoids, (−)-(10*R*)-10-hydroxy-longicyclic acid (**2**), (+)-(10*S*)-10-hydroxy-longicyclic acid (**3**), and (+)-10-oxo-longicyclic acid (**4**) by *A. niger*, and their conversion rates were 27, 23, and 30%, respectively. The metabolites suppressed the SOS-inducing activity of furylfuramide and AFB1 in the *umu* test. Compounds **1**−**4** were hardly showing a suppressive effect on *umu* gene expression of the SOS responses in *S. typhimurium* TA1535/pSK1002 against furylfuramid. However, metabolites showed a suppressive effect against AFB1. Compound **4** had gene expression by chemical mutagen AFB1, was suppressed 53% at <1.0 mM, and was the most effective compound in this experiment.

KEYWORDS: SOS-Inducing activity; furylfuramid; AFB1; *umu* test; microorganism; (+)-longicyclene; (−)-(10*R*)-10-hydroxy-longicyclic acid; (+)-(10*S*)-10-hydroxy-longicyclic acid; (+)-10-oxo-longicyclic acid

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 reactions under mild conditions and producing optically active compounds (*1*−*6*). 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. We have been studying the biotransformation of terpenoids by fungi to produce new biologically active terpenoids. In our previous papers, some terpenoids, such as (+)-fenchone, 1,4-cineole, (−)-α-bisabolol, (−)-*cis*-rose oxide, and (+)-*trans*-rose oxide, were transformed into novel terpenoids, which is stereoselective oxidation by *Aspergillus niger* (*7*−*10*).

In the past, we investigated to biotransformation of longifolenes, which are (+)-cycloisolongifolol, (−)-longifolol, (+)-α-longipinene, and (−)-isolongifolene, by a microorganism (*11*−*14*). Particularly, bioconversion of (−)-isolongifolene by *Glomerella cingulata* and the suppressive effect on *umuC* gene expression by chemical mutagens furylfuramid and AFB1 of the SOS response were studied, and the metabolite, which is (−)-isolongifolen-9-one, showed gene expression by chemical mutagens furylfuramid and AFB1, suppressing 54 and 50% at <0.5 mM, respectively (*14*). From there, we were interested in the suppressive effects of longifolenes. (+)-Longicyclene (**1**) is sesquiterpene hydrocarbon with a longifolene skeleton. In natural products, compound **1** was contained in the essential oil of the cone of *Pinus densiflora*, the roots of *Cryptomeria japonica* D. Don. (Japanese name is sugi), and *Chamaecyparis obtusa* (Sieb. et Zucc.) Endl. (Japanese name is hinoki) in Japan (*15*−*17*). 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 (*18*, *19*). 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.

As part of our continuing program to discover bioactive natural compounds, we have investigated anti-mutagenic compounds and suppressive compounds of the SOS response in plants (*20*, *21*). However, there are few reports about the suppressive effect on chemical mutagen-induced SOS response by sesquiterpenoids. Therefore, in this study, we reported the suppressive effect of sesquiterpenoids with the longifolene skeleton against chemical mutagen-induced SOS response.

MATERIALS AND METHODS

General Procedure. Nuclear magnetic resonance (NMR) was performed at 500 MHz (^{1}H) and 125 MHz (^{13}C), $CDCl_3$, with tetramethylsilane (TMS) as the internal standard. For gas chromatography (GC), an Agilent Technologies 6890N gas chromatograph equipped with a flame ionization detector, a HP-5 capillary column (30 m length, 0.25 mm inner diameter), and a split injection of 20:1 were used. Helium at a flow rate of

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

carbon number	1	2	3	4
1	1.27−1.24 (1H, m)	1.42−1.43 (1H, m)	1.41−1.44 (1H, m)	1.57−1.61 (1H, m)
2	0.94−0.95 (1H, m)	2.11 (1H, d, 5.7)	2.05 (1H, d, 5.8)	2.20−2.21 (1H, m)
3				
4	0.73 (1H, dd, 0.9, 5.5)	1.78 (1H, dd, 0.9, 5.8)	1.74−1.78 (1H, m)	1.99 (1H, dt, 1.4, 11.5)
5	1.02 (1H, d, 10.3)	1.14−1.15 (1H, m)	1.16−1.18 (1H, m)	1.25−1.27 (1H, m)
	1.73 (1H, dt, 1.4, 1.9, 10.3)	1.92 (1H, dt, 1.7, 10.9)	1.86 (1H, dd, 1.4, 1.6)	2.05 (1H, dd, 0.6, 5.8)
6	1.53−1.54 (1H, m)	1.63−1.65 (1H, m)	1.71−1.72 (1H, m)	1.63−1.66 (1H, m)
7				
8	1.22 (1H, d, 3.4)	1.39−1.41 (1H, m)	1.57−1.59 (1H, m)	2.23−2.27 (1H, m)
	1.64−1.69 (1H, m)	2.29 (1H, ddd, 3.6, 5.2, 13.6)	2.24−2.30 (1H, m)	2.78−2.84 (1H, m)
9	1.28−1.32 (1H, m)	1.37−1.38 (1H, m)	1.24−1.27 (1H, m)	1.59−1.61 (1H, m)
	1.43−1.46 (1H, m)	1.74−1.80 (1H, m)	1.68−1.72 (1H, m)	2.24−2.29 (1H, m)
10	1.34−1.39 (1H, m)	3.24−3.26 (1H, m)	3.47 (1H, d, 8.6)	
	1.07−1.12 (1H, m)			
11				
12	0.99 (3H, s)			
13	0.81 (3H, s)	1.03 (3H, s)	1.09 (1H, s)	1.15 (3H, s)
14	0.96 (3H, s)	1.01 (3H, s)	0.95 (1H, s)	1.14 (3H, s)
15	0.87 (3H, s)	1.08 (3H, s)	1.08 (1H, s)	1.19 (3H, s)

[a] Multiplicity and *J* (in parentheses) in hertz.

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 Chemistation software (Agilent Technologies, Inc.). For GC−mass spectrometry (MS), an Agilent Technologies 5973N gas chromatograph equipped with a split injector HP-5MS capillary column (30 m length, 0.25 mm inner diameter) was combined by direct coupling to a Agilent Technologies 5973N 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 electron impact (EI) energy was 70 eV, and the ion source temperature was 280 °C. IR spectra were obtained with a Perkin-Elmer 1760X spectrometer. $CHCl_3$ was used as a solvent. Thin-layer chromatography (TLC) used silica gel 60 F254 precoated (layer thickness = 0.25 mm, Merck), and column chromatography (CC) used silica gel developed with a hexane−EtOAc gradient.

Materials. (+)-Longicyclene used for substance was purchased from Fluka (Tokyo, Japan).

Furylfuramide and aflatoxin B_1 (AFB1) were purchased from Wako Pure Chemical Co. (Osaka, Japan).

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

Preculture of *A. niger*. Spores of *A. niger*, 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 at pH 7.2) in a shaking flask at 27 °C for 1 day.

Time-Course Experiment. Precultured *A. niger* was transferred into a culture medium (20 mL in a 50 mL Petri dish) and incubated for 1 day (until mycelia occupied 60−80% of the surface area of a culture medium) under the stationary culture. After the growth of *A. niger*, compound **1** (0.3 mg/mL) was added to the medium and the culture was continued for 10 days, salted with NaCl, and then extracted with EtOAc. The extract was analyzed by GC. The ratios between the substrate compound **1** and metabolic products were determined on the basis of the peak areas of GC and GC−MS.

Isolation of Metabolites. (+)-Longicyclene was incubated with *A. niger* as described above for 10 days into 2 L (20 mL in a 50 mL Petri dish). After the biotransformation for 10 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 (752 mg). The extract was a chromatographer on silica-300 columns with hexane−EtOAc; compound **1** (150 mg) was recovered. Metabolites **2** (36 mg), **3** (28 mg), and **4** (83 mg) were isolated.

***umu* Test.** Method of the *umu* test for detecting the SOS-inducing activity of chemicals was carried out according to Miyazawa et al. using *Salmonella typhimurium* TA1535/pSK1002, whose plasmid pSK1002 carries a *umuC*−*lacZ* fused gene.

Briefly, the overnight culture of the 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 subdivide into 2.1 mL portions in test tubes, and test compound [50 μL, in dimethylsulfoxide (DMSO)], 0.1 m phosphate buffer (300 μL, pH 7.4), and mutagens, which are furylfuramid or AFB1 (50 μL, in DMSO), were added to each tube. In the case of AFB1, the S9 mix was added in each tube instead of phosphate buffer. As a positive control, an equivalent volume of DMSO was added instead of the test compound, whereas with negative controls, an equivalent volume of DMSO was added instead of both the 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 phosphate-buffered saline (PBS). The optical densities of the suspensions at 600 nm were recorded with one portion (0.25 mL), while the rest of the cell suspensions were used to measure the level of intracellular β-galactosidase activity using the method by Miller.

(−)-(10*R*)-10-Hydroxylongicyclic Acid (2). Colorless powder. $[\alpha]^{21.5}_D$ −1.7 (*c* 0.5, $CHCl_3$). High-resolution mass spectrometry−fast atom bombardment (HRMS−FAB) (*m*/*z*): 251.1646 $[M + H]^+$ (calcd for $C_{15}H_{23}O_3$, 251.1648). Electron-impact mass spectrometry (EIMS) *m*/*z* (relative intensity): $[M]^+$ 250 (5), 232 (15), 204 (12), 162 (48), 108 (59), 91 (72), 69 (100), 41 (86). IR (KBr) ν_{max} (cm^{-1}): 3407, 2962, 2930, 1677, 1434. ^{1}H and ^{13}C NMR spectral data are shown in **Tables 1** and **2**.

(+)-(10*S*)-10-Hydroxylongicyclic Acid (3). Colorless powder. $[\alpha]^{21.5}_D$ +1.6 (*c* 0.5, $CHCl_3$). HR-EIMS (*m*/*z*): 250.1645 $[M]^+$ (calcd for $C_{15}H_{22}O_3$, 250.1647). EIMS *m*/*z* (relative intensity): $[M]^+$ 250 (4), 232 (15), 204 (14), 143 (55), 105 (68), 91 (100), 69 (94), 41 (99). IR (KBr) ν_{max} (cm^{-1}): 3420, 2960, 2923, 1682, 1456. ^{1}H and ^{13}C NMR spectral data are shown in **Tables 1** and **2**.

(+)-10-oxo-Longicyclic Acid (4). Colorless powder. $[\alpha]^{21.5}_D$ +22.3 (*c* 0.5, $CHCl_3$). HR-EIMS (*m*/*z*): 248.1416 $[M]^+$ (calcd for $C_{15}H_{20}O_3$, 248.1413). EIMS *m*/*z* (relative intensity): $[M]^+$ 248 (51), 230 (34), 202 (16), 123 (69), 107 (77), 93 (100), 69 (94), 41 (90). IR (KBr) ν_{max} (cm^{-1}): 2965, 2926, 1698, 1674. ^{1}H and ^{13}C NMR spectral data are shown in **Tables 1** and **2**.

RESULTS AND DISCUSSION

Biotransformation of (+)-Longicyclene (1) by *A. niger*. Biotransformation of (+)-longicyclene (**1**) by *A. niger* was investigated.

Table 2. ^{13}C NMR Spectral Data for Compounds **1**−**4** (δ, TMS, in $CDCl_3$ at 125 MHz)[a]

carbon number	1	2	3	4
1	58.3	57.1	57.4	55.7
2	22.9	26.4	26.3	29.1
3	24.4	30.8	33.1	31.1
4	17.7	29.4	27.2	25.5
5	33.8	34.0	33.9	33.9
6	42.1	42.7	42.2	44.5
7	46.1	45.0	44.8	45.0
8	38.8	35.0	30.3	38.4
9	20.7	30.4	28.4	32.5
10	41.7	79.8	76.7	215.9
11	33.6	37.9	37.8	46.8
12	10.5	178.5	178.7	179.2
13	23.0	22.8	24.6	22.1
14	28.6	25.0	28.1	26.0
15	33.0	30.5	23.3	26.5

[a] Chemical shifts in parts per million. Multiplicities were determined by the distortionless enhancement by polarization transfer (DEPT) pulse sequence.

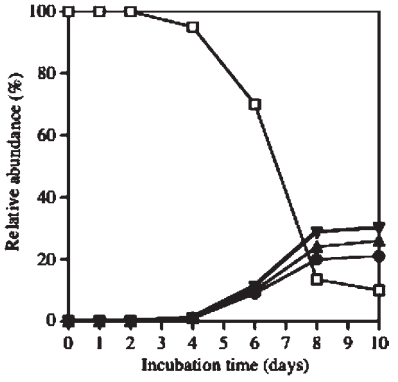

Figure 1. Time course of the biotransformation of compound **1** by *A. niger*: (□) (+)-longicyclene (**1**), (▲) (−)-(10*R*)-10-hydroxylongicyclic acid (**2**), (●) (+)-(10*S*)-10-hydroxylongicycylic aid (**3**), and (▼) (+)-10-oxolongicyclic acid (**4**).

For time-course experiments, a small amount of compound **1** was incubated with *A. niger* for 10 days. Compound **1** was transformed to three metabolites, which were detected by TLC, GC, and GC−MS analyses. **Figure 1** shows the time course for the appearance of the metabolites; about 80% of compound **1** was metabolized after 10 days. The conversion rate of metabolites **2**, **3**, and **4** was 27, 23, and 30%, respectively. These products were not detected on TLC, GC, and GC−MS analyses of the culture of *A. niger* to which no substrate was fed.

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

Compound **2** was determined from the following MS, IR, and NMR data. Compound **2** had molecular formula $C_{15}H_{22}O_3$ from HRMS-FAB (*m*/*z*) 251.1646 $[M + H]^+$ (calcd for $C_{15}H_{23}O_3$, 251.1648). IR and ^{13}C NMR spectra of **2** showed the presence of a hydroxyl group [IR, 3432 cm^{-1}; δ_C, 79.8 (CH)] and a carboxyl group [IR, 1683 cm^{-1}; δ_C, 178.5 ppm (C)]. ^1H and ^{13}C NMR signals were similar to that of the substrate, except for the existence of a new methine group and non-protonated carbon and disappearance of a methylene group and methyl group. ^1H NMR provides evidence for the presence of three methyl groups. Compound **2** was confirmed by assignment of the NMR spectra using

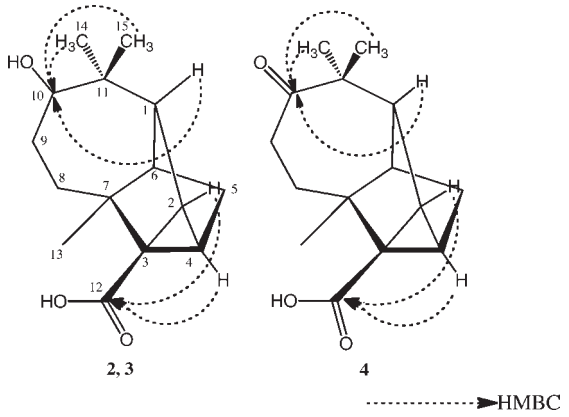

Figure 2. Key HMBC spectra of compounds **2**, **3**, and **4**.

two-dimensional techniques [correlation spectroscopy (COSY), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple-bond connectivity (HMBC)]. In the characteristic HMBC spectrum, correlations were observed of (i) H-14, H-15, and H-1/C-10 and (ii) H-2 and H-4/C-12 (**Figure 2**). Therefore, compound **2** was produced by hydroxylation at the C-10 position and carboxylation at the C-12 position of compound **1**. Furthermore, the absolute configuration of compound **2** was determined by the modified Mosher method. Compound **2** was esterified with (±)-MTPA−Cl in CH_2Cl_2 and DMAP to give (−)-MTPA ester and (+)-MTPA ester, respectively. The $\Delta\delta$ values [$\delta(-) - \delta(+)$] indicated that the absolute configuration of C-10 was *R*. The specific rotation shows the (−) form. From these data, it was concluded that the structure of compound **2** is (−)-(10*R*)-10-hydroxy-longicyclic acid.

The second metabolite, compound **3**, had molecular formula $C_{15}H_{22}O_3$ from HR-EIMS (*m*/*z*): 250.1580 $[M]^+$ (calcd for $C_{15}H_{22}O_3$, 250.1647). The IR and ^{13}C NMR spectrum of compound **3** showed the presence of a hydroxyl group [IR, 3420 cm^{-1}; δ_C, 76.7 (CH)] and a carboxyl group [IR, 1682 cm^{-1}; δ_C, 178.7 ppm (C)]. ^1H and ^{13}C NMR signals and two-dimensional techniques (COSY, HMQC, and HMBC) were similar to that of compound **2**. Therefore, compound **3** was an epimer of compound **2**. Compound **3** was produced by hydroxylation at the C-10 position and carboxylation at the C-12 position of compound **1**. The configuration of C-10 of compound **3** as *S* was inferred from the modified Mosher method. The specific rotation shows the (+) form. From these data, it was concluded that the structure of compound **3** is (+)-(10*S*)-10-hydroxy-longicyclic acid.

The third metabolite, compound **4**, had molecular formula $C_{15}H_{20}O_3$ from HR-EIMS (*m*/*z*): 248.1416 $[M]^+$ (calcd for $C_{15}H_{20}O_3$, 248.1413). The IR and ^{13}C NMR spectrum of compound **4** showed the presence of a carbonyl group [IR, 1674 cm^{-1}; δ_C, 215.9 (C)] and a carboxyl group [IR, 1698 cm^{-1}; δ_C, 179.2 ppm (C)]. ^1H and ^{13}C NMR signals were similar to that of the substrate, except for the existence of a new methine group and non-protonated carbon and disappearance of a methylene group and methyl group. ^1H NMR provided evidence for the presence of three methyl groups. This was confirmed by assignment of the NMR spectra using two-dimensional techniques. In the characteristic HMBC spectrum, correlations were observed of (i) H-1, H-14, and H-15/C-10 and (ii) H-2 and H-4/C-12 (**Figure 2**). Therefore, compound **4** was produced by oxolation at the C-10 position and carboxylation at the C-12 position of compound **1**. The specific rotation shows the (+) form. From these data, it was concluded that the structure of compound **4** is (+)-10-oxo-longicyclic acid.

To clarify the metabolic pathway in the biotransformation of compound **1** by *A. niger*, small amounts of compounds **2**, **3**, and **4**

were incubated with *A. niger* for 10 days, respectively. As a result, compounds **2** and **3** were bioconverted to compound **4** by *A. niger*, and compound **4** was not bioconverted. Therefore, the metabolic pathways in the biotransformation of compound **1** by *A. niger* were shown in **Figure 3**.

In conclusion, compound **1** was oxidized to the C-10 position of the seven-membered ring and C-12 position of the end of the methyl group by *A. niger*. The reaction in the C-10 position is oxidized to ketone, and an intermediate hydroxide is not stereoselective. In addition, the reactive site was the α position of geminal dimethyl in the seven-membered ring. Reportedly, (−)-isolongifolol was biotransformed into 10α-hydroxyisolongifolol and 9α-hydroxyisolongifolol by *A. niger* (*22*). This report showed that the reactive site was the α and *β* positions of geminal dimethyl in the seven-membered ring.

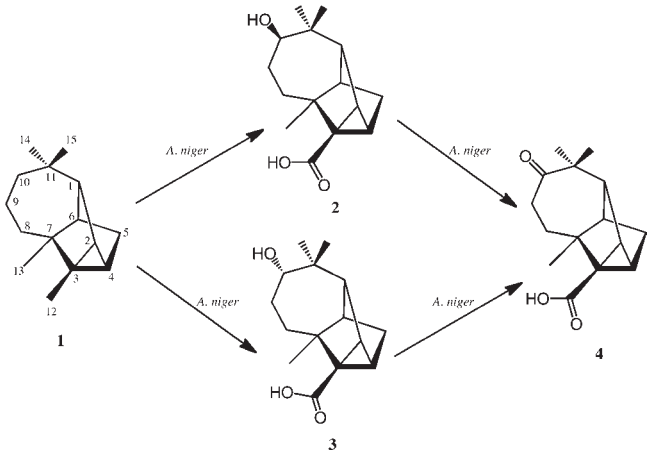

Figure 3. Biotransformation of (+)-longicyclene (**1**) by *A. niger*.

It is suggested that *A. niger* has the ability of oxidation at the α position of geminal dimethyl on the seven-membered ring in longifolene skeleton.

Suppressive Effect on *umuC* Gene Expression by Chemical Mutagens Furylfuramid and AFB1. The suppressive effects of compounds **1**−**4** were determined in the *umu* test, and cell growth was unaffected at a concentration of these compounds at experimental conditions. Compounds **1**−**4** were hardly showing a suppressive effect on *umu* gene expression of the SOS responses in *S. typhimurium* TA1535/pSK1002 against furylfuramid. As shown in **Figure 4**, metabolites showed a suppressive effect on *umu* gene expression of the SOS responses in *S. typhimurium* TA1535/pSK1002 against AFB1. Compound **1** suppressed 56% of the SOS-inducing activity at concentrations less than 1.0 mM, and the IC_{50} value was 0.77 mM. Compound **2** suppressed 54% of the SOS-inducing activity at concentrations less than 0.5 mM, although this compound showed toxicity at 1.0 mM. Compounds

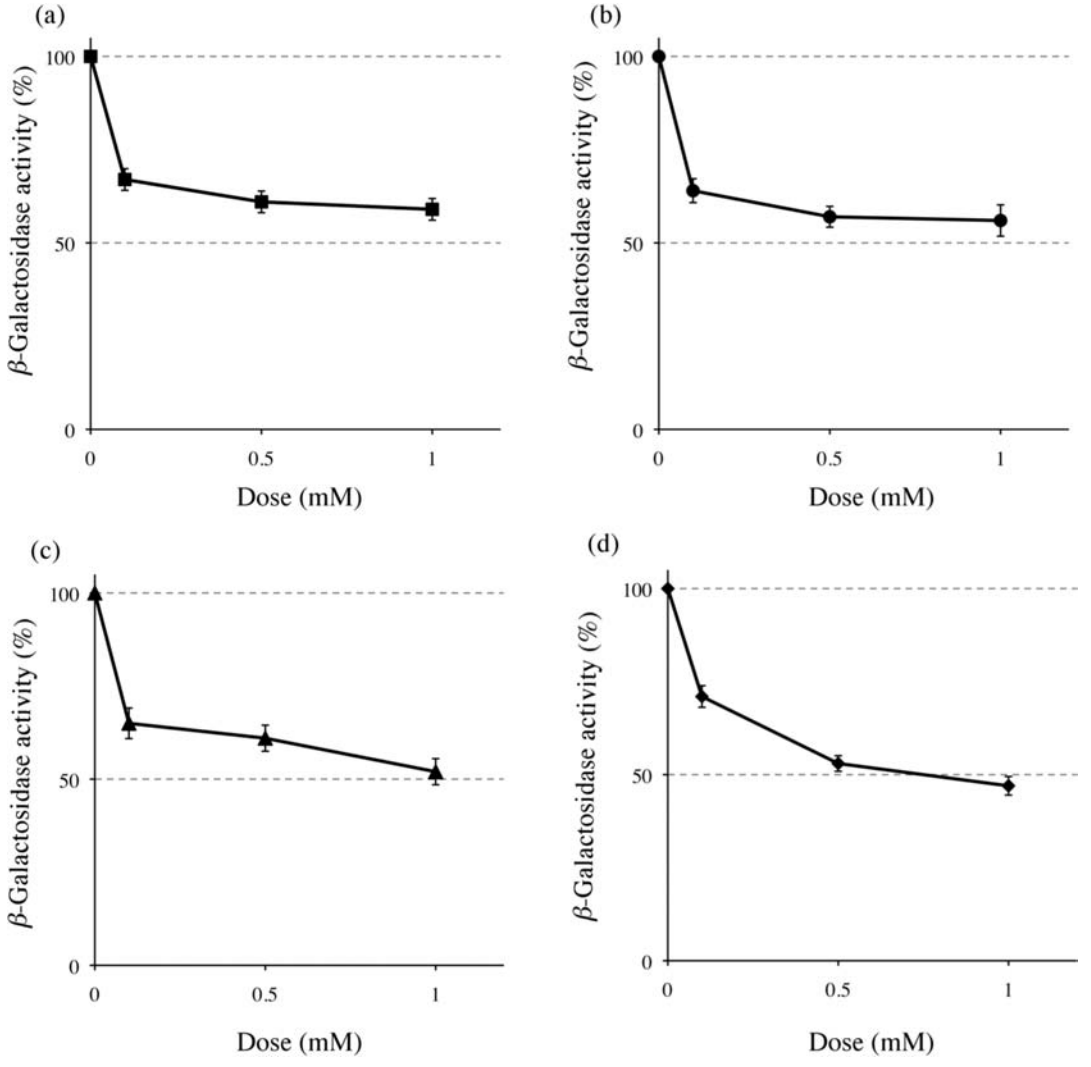

Figure 4. Suppression of AFB1-induced SOS responses by compounds **1**−**4**: (a) effect of compound **1**, (b) effect of compound **2**, (c) effect of compound **3**, and (d) effect of compound **4**. AFB1 (33 μg/mL in DMSO) was added at 10 μL. Data represent the mean ± standard deviation (SD) from four experiments.

3 and **4** suppressed 38.8 and 40.2% of the SOS-inducing activity at concentrations less than 1.0 mM, respectively. In human levier, AFB1 is metabolically converted by CYP3A4 to carcinogenic *exo*-AFB1-epoxide, which forms mutagenic AFB1-DNA adducts (*23*). Longifolenes have the potential of inhibition of CYP3A4 because AFB1 is transformed to carcinogenic *exo*-AFB1-epoxide catalyzed by CYP3A4.

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