Antimutagenic Activity of (+)-Polyalthic Acid from *Vitex rotundiforia*

Mitsuo Miyazawa,*,† Hideo Shimamura,† Sei-ichi Nakamura,‡ and Hiromu Kameoka†

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka-shi, Osaka 577, Japan, and Osaka Prefectural Institute of Public Health, Nakamichi-1, Higashinari-ku, Osaka 537, Japan

A methanol extract from *Vitex rotundiforia* showed a suppressive effect on *umu* gene expression of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1)), which requires liver metabolizing enzymes. The methanol extract from *V. rotundiforia* was re-extracted with dichloromethane, *n*-butanol, and water, respectively. A suppressive compound in the dichloromethane extract fraction was isolated by SiO_2 column chromatography and identified as (+)-polyalthic acid by EI-MS and ^1H and ^{13}C NMR spectroscopy. (+)-Polyalthic acid suppressed the SOS-inducing activity of Trp-P-1 in the *umu* test. Gene expression was suppressed 90% at <0.32 μmol/mL, and the ID_{50} value was 0.19 μmol/mL. (+)-Methyl polyalthate also suppressed the SOS-inducing activity of Trp-P-1 in the *umu* test. Gene expression was suppressed 57% at <0.32 μmol/mL, and the ID_{50} value was 0.29 μmol/mL. (+)-Polyalthic acid and (+)-methyl polyalthate were also assayed with the mutagens 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide) and activated Trp-P-1, but these compounds did not show a suppressive effect on the SOS induction of these mutagens. The antimutagenic activities of (+)-polyalthic acid and (+)-methyl polyalthate against Trp-P-1, furylfuramide, and activated Trp-P-1 were tested by an Ames test using *S. typhimurium* TA100. The results indicated that (+)-polyalthic acid suppressed the mutagenicity of Trp-P-1 and the mutagenicity of activated Trp-P-1 and (+)-methyl polyalthate suppressed the mutagenicity of Trp-P-1.

Keywords: *Vitex rotundiforia; (+)-polyalthic acid; Verbenaceae; antimutagenic activity; umu test; Ames test*

INTRODUCTION

Vitex rotundiforia (Verbenaceae) is widely distributed on the sea coast in Asia. The fruits of this plant have been used in the treatment of headaches; and the plant is known as a rich source of iridoid glucoside (Kouno et al., 1988) and agnuside (Hansel et al., 1965). Previously, the presence of the diterpenoids rotundifuran and perrotundifuran (Asaka et al., 1973; Hirotsu et al., 1973; Kondo et al., 1986) has been reported in Verbenaceae.

In evaluating 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. Sandenes et al. (1992) reported studies on the mutagenicity of crude Senna and Senna glycosides. Kakinuma et al. (1984) reported antimutagenic diterpenoids from the crude drug Isodonis Herba.

The *umu* test system was developed to evaluate the genotoxic activities of a wide variety of enviromental 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). 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., 1995a,b), we found that the methanol extract of *V. rotundiforia* (Mankeishi in Japanese) exhibited a suppression of the SOS-inducing activity of Trp-P-1. In this paper, we report the isolation and identification of the antimutagenic compound contained in *V. rotundiforia*.

MATERIALS AND METHODS

General Procedure. Electron-impact mass spectra (EI-MS) were obtained on a Shimadzu QP1000A mass spectrometer. Infrared spectra were determined with a Perkin-Elmer 1760-x IR 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 (δ0.00) for ^1H NMR spectra measured in $CDCl_3$. This solvent was also used for ^{13}C NMR spectra. Specific rotation was determined with a JASCO DIP-140 digital polarimeter.

Materials. Commercially available air-dried powder of *V. rotundiforia* (Mankeishi) was obtained from Takasago Yakugiyo Co. (Osaka, Japan). 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 whose plasmid pSK1002 carries a *umuC'-'lacZ* fused gene. The SOS-inducing potency is estimated by the measurement of the level of *umu* operon expression in terms of cellular β-galactosidase activity.

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

Suppression of Metabolic Activation of Trp-P-1. The suppressive effect of (+)-polyalthic acid (**1**) and (+)-methyl

* Author to whom correspondence should be addressed (telephone +81-6-721-2332; fax +81-6-727-4301).

† Kinki University.

‡ Osaka Prefectural Institute of Public Health.

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Table 1. Suppressive Effect of *V. rotundiforia* Fractions on Trp-P-1[a] Using *S. typhimurium* TA1535/pSK1002

		dose response[c]			
sample	control[b]	200 μg/mL	100 μg/mL	50 μg/mL	0 μg/mL
MeOH extract	240.3	570.0	635.2	716.8	921.1
CH_2Cl_2 fraction	131.7	250.3	310.4	386.1	405.3
n-BuOH fraction	131.7	325.5	358.9	382.0	405.3
water fraction	131.7	401.9	417.6	393.7	405.3
fraction 1	154.1	598.2	615.2	612.3	628.0
fraction 2	154.1	224.3	346.4	563.1	628.0
fraction 3	154.1	453.2	562.4	603.5	628.0
fraction 4	154.1	589.3	621.3	612.7	628.0
fraction 5	185.7	503.5	544.2	812.2	598.9
fraction 6	185.7	255.2	310.5	480.6	598.9
fraction 7	185.7	235.2	302.8	493.7	598.9
fraction 8	185.7	504.2	563.2	586.2	598.9
fraction 9	185.7	563.2	542.3	563.2	598.9
fraction 10	185.7	532.2	596.5	573.4	598.9

[a] Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL. [b] Control was a treatment without Trp-P-1. [c] β-Galactosidase activity (units).

polyalthate (**1-Me**) on metabolic activation of Trp-P-1 was examined as follows: 100 μL of Trp-P-1 (100 μg/mL in distilled H_2O) was added to each tube. Then 100 μL of a test sample (10 mg/mL in DMSO), 200 μL of S9-mix, and 800 μL of 0.1 M phosphate buffer were added to each tube. To obtain dose–response data, test samples were evaluated at dose levels of 2.6, 1.3, 0.53, and 0.26 μmol/mL. After 20 min of incubation at 37 °C with shaking, 1.0 mL of acetone was added and the mixture was centrifuged to collect supernatants, which were evaporated to dryness under reduced pressure. The residue was diluted to 2.0 mL with distilled H_2O. The mixture suspension of activated Trp-P-1 was assayed by *umu* test. The culture was divided into 2.0-mL portions in test tubes, and 500 μL of the mixture suspension was added. The dose values that made use of suppression of metabolic activation corresponded to 0.32, 0.16, 0.06, and 0.03 μmol/mL in the *umu* test.

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

Purification of the Suppressive Compound 1. The dry powder (5 kg) of *V. rotundiforia* was refluxed with methanol for 12 h to give a methanol extract (350 g). This extract was suspended in water (2 L) and partitioned between dichloromethane (1 L) and water, then *n*-butanol (1 L) and water, successively. Each soluble fraction was concentrated under reduced pressure to give dichloromethane (120 g), *n*-butanol (110 g), and water (105 g) fractions. To pursue the compound responsible for the suppression of the SOS-inducing activity, these fractions were tested. As shown in Table 1, the dichloromethane fraction had positive activity, whereas the *n*-butanol and water fractions did not show activity. The dichloromethane fraction was fractionated to fractions 1–4 by SiO_2 column chromatography (13 × 50 cm and 3 kg of silica gel) with hexane, ether, ethyl acetate, and methanol as eluents. Fraction 2 showed suppression of the SOS-inducing activity of Trp-P-1 in the *umu* test. Therefore, fraction 2 was fractionated to fractions 5–10 by SiO_2 column chromatography (13 × 50 cm and 1 kg of silica gel), and fraction 6 and 7 were combined and repeatedly fractionated by SiO_2 column chromatography. Finally, suppressive compound **1** (3.2 g) was isolated.

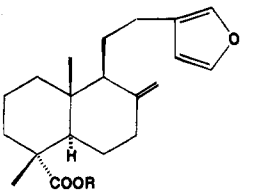
Suppressive Compound 1. Compound **1** was crystalline: mp, 110–112 °C; $[\alpha]_D^{20}$ +23.3° (ethanol; *c* 1.0); MS *m/z* 316 (M^+), 271 (3%), 189 (9%), 121 (53%), 95 (75%), 81 (100%); IRγ_{max} KBr 2936, 1694, 1499, 1464, 1386, 1276, 1166, 1024 cm^{-1}; ^1H NMR identical with that of (+)-polyalthic acid (Ohta et al., 1987). The suppressive compound **1** was identified as (+)-polyalthic acid from these spectral data and physical properties.

Methyl Ester of Suppressive Compound 1 (1-Me). **1-Me** was obtained by the reaction with diazomethane. This compound was a colorless oil: $[\alpha]_D^{20}$ +20.38° (ethanol; *c* 1.55); MS *m/z* 330 (M^+), 271 (16%), 236 (31%), 189 (18%), 121 (100%), 81 (82%); IRγ_{max} KBr 2943, 2858, 1730, 1645, 1453, 893, 872 cm^{-1}; ^1H NMR identical with that of (+)-methyl polyalthate (Ohta et al., 1987). **1-Me** was identified as (+)-methyl polyalthate from these spectral data and physical properties.

RESULTS

Fractionation of the Extract from *V. rotundiforia* and Isolation of (+)-Polyalthic Acid (1). The methanol 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. As shown in Table 1, the methanol extract from *V. rotundiforia* showed a suppressive effect on *umu* gene expression of the SOS responses in *S. typhimurium* TA1535/pSK1002 against Trp-P-1, which requires liver metabolizing enzymes. To prepare the suppressive fraction, fractionation of the methanol extract from *V. rotundiforia* was carried out as described under Materials and Methods.

Structure Determination of 1 and 1-Me. Compound **1** gave a molecular ion as a base peak in the EI mass spectrum at *m/z* 316. The ^1H NMR spectrum of **1**



1 : R=H
1-Me: R=Me

confirmed the presence of a methyl group at δ 0.96 (Me-10) and 1.16 (Me-4), and the signals at δ 4.56 (Ha-12) and 4.74 (Hb-12) could be assigned at the two exocyclic vinyl hydrogens. The signals at δ 6.26 (H-4″), 7.20 (H-2″) and 7.34 (H-5″) could be assigned to the furan ring. Thus, **1** was identified as (+)-polyalthic acid.

Compound **1-Me** showed a molecular ion at *m/z* 330. The ^1H NMR spectra of **1-Me** indicated the appearance of a δ 3.65, which further confirmed the structure of **1-Me** as (+)-methyl polyalthate.

Inhibition of the SOS-Inducing Activity of 1 and 1-Me. The suppressive effect of **1** and **1-Me** were determined in the *umu* test. As shown in Table 2, **1** and **1-Me** exhibited inhibition of the SOS induction of Trp-P-1. Compound **1** suppressed 90% of the SOS-inducing activity at concentrations <0.32 μmol/mL, and the ID_{50} value was 0.19 μmol/mL. Compound **1-Me** suppressed 57% of the SOS-inducing activity at concentrations <0.32 μmol/mL, and the ID_{50} value was 0.29 μmol/mL. Although both **1** and **1-Me** showed suppression of the SOS-inducing activity of Trp-P-1, which requires metabolizic activations, neither of them showed suppression of the activity of furylfuramide and activated Trp-P-1.

Suppressive Effect of 1 and 1-Me on Metabolic Activation of Trp-P-1. The suppressive effects of **1** and **1-Me** on metabolic activation of Trp-P-1 were tested by the *umu* test. The value of β-galactosidase activity observed in the absence of these compounds was 674.9 for activated Trp-P-1. As shown in Figure 1, metabolic activation was suppressed by **1** and **1-Me**, and inhibition was parallel with mutagenic activity. This result sug-

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

chemical	Trp-P-1	furylfuramide	activated Trp-P-1	control[d]	dose response[e] 0.32 μmol/mL	0.16 μmol/mL	0.06 μmol/mL
1	598.9			185.7	227.1	427.4	528.7
		821.9		189.6	878.8	897.8	840.9
			665.1	189.6	570.0	622.1	646.1
1-Me	598.9			185.7	363.4	503.9	582.4
		821.9		189.6	720.7	796.6	824.3
			665.1	189.6	622.3	636.6	655.6

[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] Activated Trp-P-1 (prepared from 6.7 μg Trp-P-1) was added at 50 μL. [d] β-Galactosidase activity (units).

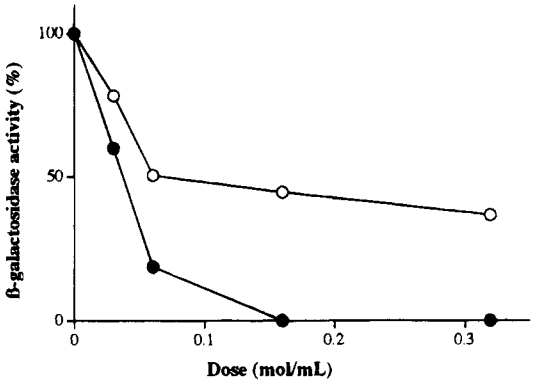


Figure 1. Suppressive effect of **1** and **1-Me** on metabolic activation of Trp-P-1. Key: (●) 1; (○) **1-Me**. The metabolic activation of Trp-P-1 was assayed as follows: The procedure consisted of treatment of Trp-P-1 and test sample with S9-mix, acetone precipitation of the proteins, and evaporation of the supernatant. Activity of the direct mutagen was assayed on *S. typhimurium* TA1535/pSK1002. The value of β-galactosidase activity measured in the absence of test sample was 674.9.

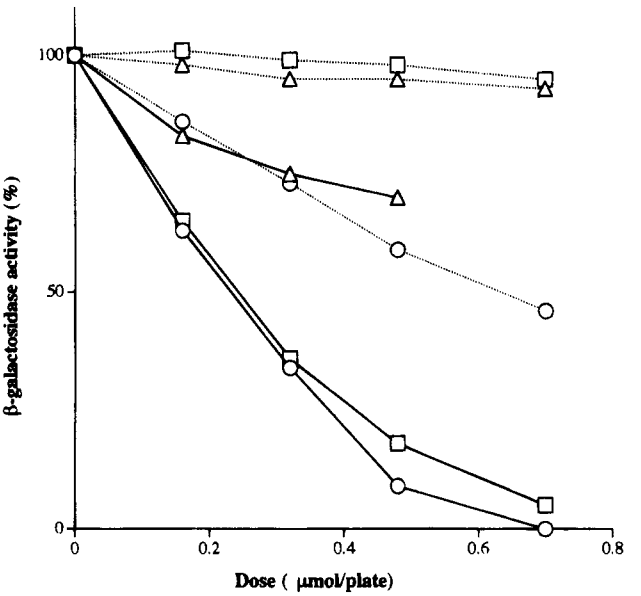


Figure 2. Effect of **1** and **1-Me** on the mutagenicity of Trp-P-1, furylfuramide, and activated Trp-P-1 in *S. typhimurium* TA100. Key: (—) 1; (···) 1-Me; (○) Trp-P-1; (△) furylfuramide; (□) activated Trp-P-1. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL/plate, furylfuramide (1 μg/mL in DMSO) was added at 50 μL/plate, and activated Trp-P-1 was added at 50 μL/plate. In case of furylfuramide, **1** was toxic at <0.70 μmol/plate.

gests that the inhibition of the SOS-inducing activity of Trp-P-1 is due to the inhibition of metabolic activation.

Antimutagenic Activity of 1 and 1-Me. The antimutagenic activity of these compounds against Trp-P-1 was also demonstrated by the Ames test using *S. typhimurium* TA100. As shown in Figure 2, **1** suppressed the mutagenicity of Trp-P-1 completely at 0.70 μmol/plate, and the ID_{50} value was 0.22 μmol/plate. Compound **1** suppressed 90% of the mutagenicity of Trp-P-1 at 0.70 μmol/plate, and the ID_{50} value was 0.24 μmol/plate. In the case of furylfuramide, **1** showed the toxicity at a concentration of 0.7 μmol/plate and ~30% suppression of the mutagenicity at 0.48 μmol/plate. Compound **1-Me** suppressed 53% of the mutagenicity of Trp-P-1 at 0.70 μmol/plate, and the ID_{50} value was 0.63 μmol/plate. This compound showed little suppression of the mutagenicity of furylfuramide and activated Trp-P-1 similar to the *umu* test. From the result of the *umu* test, **1** did not suppress the SOS-inducing activity of activated Trp-P-1, but it did suppress the mutagenicity of activated Trp-P-1 in the Ames test.

DISCUSSION

The antimutagenic compound in *V. rotundiforia* was clearly identified as due to **1**. This compound had a suppressive effect on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against Trp-P-1, which requires liver metabolizing enzymes. Compounds **1** and **1-Me** were examined for their ability to suppress the metabolic activation of Trp-P-1 (Figure 1). As shown in Figure 1, the metabolic activation was suppressed by **1** and **1-Me**, and the suppression was parallel with that of the suppressive effect.

In the Ames test, **1** and **1-Me** suppressed the mutagenicity of Trp-P-1 similar to the *umu* test, whereas **1** suppressed the mutagenicity of activated Trp-P-1 (Figure 2). This result was different from that of the *umu* test. The difference between the result of the Ames and the *umu* tests may be caused by the difference of these methods. The principle of the *umu* test is based on the ability of DNA-damaging agents, most of which are potential mutagens and carcinogens, to induce the *umu* operon. A plasmid (pSK1002) carrying a fused gene (*umuC'-'lacZ*) was introduced into *S. typhimurium* TA1535. The strain TA1535/pSK1002 enabled us to monitor the levels of *umu* operon expression by measuring the β-galactosidase activity in the cells produced by the fusion gene. The Ames test is based on His^+ colonies as spontaneous His^+ revertant colonies, and the Ames tester strain TA100 carries a *uvrB* mutation. From the results of both the *umu* test and the Ames test, it may be expected that a correlation between the mutagens and **1** is the suppression of the metabolic activation of S9-mix and other functions, and **1-Me** is the suppression of metabolic activation by the S9-mix. Consequently, it was suggested that **1** is an antimutagenic compound and **1-Me** is a desmutagenic compound.

The presence of the diterpenes, rotundifuran, prerotundifuran, vitexilactone, and previtexilactone from *V. rotundiforia* has been reported (Asaka et al., 1973; Kondo et al., 1986). In the investigation on the other constituents of same plants, several compounds have been isolated; for examples, iridoid glucoside, flavone, and benzofuran (Tada et al., 1984; Kondo et al., 1986; Kouno et al., 1988). Previously, the stereomer of **1** has also been isolated from several plants; for examples, *Polyalthia fragrans* (Anonaceae), *Daniellia oliveri* (Amherstiae), and *Pinus lambertiana* (Pinaceae; Gopinath et al., 1961; Dauben et al., 1966; Haeuser et al., 1970). Compound **1** has been isolated from leaves of *Sequoia semperivirens* (Taxodiaceae) and exhibits a repellent activity against the sea snail *Monodonta neritoides* (Ohta et al., 1987). However, no reports on antimutagenic or desmutagenic activity of **1** and **1-Me** have appeared.

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