Suppression of Chemical Mutagens-Induced SOS Response by Phenolic Acids from Black Rice Bran Using Salmonella typhimurium TA1535/pSK1002 umu Test

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Abstract: A methanol extract from black rice bran showed a suppressive effect of the SOS-inducing activity on the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide) in the Salmonella typhimurium TA1535/pSK1002 umu test. The methanol extract was re-extracted with hexane, chloroform, ethylacetate, butanol and water. The ethylacetate fraction showed a suppressive effect. Suppressive compounds in an acidic fraction of the ethylacetate fraction were isolated by silica gel column chromatography and identified as vanillic acid (1) and protocatechuic acid (2) by GC, GC/MS, IR and 1H and 13C NMR spectroscopy. Compounds 1 and 2 suppressed the furylfuramide-induced SOS response in the umu test. Compounds 1 and 2 suppressed 37.7 and 44.5% of the SOS-inducing activity on furylfuramide at a concentration of 1.20 μmol/mL. These compounds were assayed with other mutagens, 4-nitroquinolin 1-oxide (4NQO) and N-methyl-N-bral-N-nitrosoguanidine (MNNG), which do not require liver metabolizing enzymes. In addition, compounds 1 and 2 were assayed with aflatoxin B1 (AfB1) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), which require liver metabolizing enzymes. These compounds showed suppressive effects of the SOS-inducing activity against furylfuramide, 4NQO, MNNG, AfB1 and Trp-P-1. To research the structure-activity relationship, veratric acid (3) as a similar compound of 1 and methyl esters of 1, 2 and 3 (1Me, 2Me and 3Me) were also assayed with all chemical mutagens. Compounds 1Me, 2Me and 3Me exhibited stronger suppressive effects of the SOS-inducing activity against all chemical mutagens than 1, 2 and 3.

Key words: black rice bran, vanillic acid, protocatechuic acid, SOS response, umu test

1 Introduction

In the evaluation of the carcinogenicity or mutagenicity caused by 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 (1), it has been shown that ordinary diets contain many kinds of mutagens and antimutagens.

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In particular, the *umu* test system was developed as a simple, but sensitive, tool to evaluate the genotoxic activities of a wide variety of environmental carcinogens and mutagens, using the expression of the *SOS* genes to detect DNA-damaging agents in the *S. typhimurium* (2,3). The *umu* test detects the induction of the *SOS* response following treatment of the *S. typhimurium* strain TA1535 with test compounds. This strain carries the plasmid pSK1002 in which the *umuC* gene is fused inflame to the *lacZ* gene. The *SOS*-inducing potency of test compounds would therefore be estimated by the measurement of induction of the level of *umu* operon in terms of intracellular β-galactosidase activity.

The *SOS* response appears to be induced by an alteration in DNA synthesis, either directly by DNA damage blocking to the replication fork or indirectly by antibiotics, such as novobiocin, that inhibit DNA synthesis. The *SOS* regulatory system is controlled in part by the interplay of two proteins—the LexA protein, which represses asset of unlinked genes during normal cell growth, and the RecA protein, which is required in *vivo* for inactivation of LexA protein after treatments that derepress the system by DNA damaging its metabolism (4-6).

Black rice (*Oryza Sativa* L. Indica), having dark purple-colored grains, is mainly cropped in the South Asia and Mainland China. It is broadly known as an enriched rice with medicinal effects. It is superior to robust nourishment and acts as enhancer of spleen, liver, stomach and intestine and as a hematopoietic agent in pharmacy. Anthocyanin pigments cyanidin 3-glucoside and peonidin 3-glucoside were isolated from this plant (7). Besides, it contains a lot of proteins, various amino acids and vitamins. As for mineral, it contains iron and calcium twice or three times as much as polished rice does. Various compounds, such as pheno
cic acids, ferulic acid, vanillic acid and protocatechuic acid, act as an antioxidant (12-16), as a radical scavenger (13), as an inhibitor of hepatic cytochrome P450 (17), as an antibacterial agent (18) and also as an anti-inflammatory agent (19,20). In our search for new naturally occurring antimutagenic compounds in plants, with a history of safe use as Chinese crude drugs (21,22), we found that the methanol extract of black rice bran exhibited a suppression of the furylfuramide-

Induced SOS response. In this paper, we report the isolation and identification of the suppressive compounds on SOS response against mutagens in black rice bran.

### 2 Experimental

#### 2.1 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 on a Hewlett-Packard 5972 series mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a column (HP-5MS, 30 m × 0.25 mm i.d.). IR spectra were determined with a FT/IR-470 Plus Fourier Transform Infrared Spectrometer. Nuclear magnetic resonance (NMR) spectra (δ, J in hertz) were recorded on a JEOL GSX 500 NMR spectrometer. Tetramethysilane (TMS) was used as the internal reference (δ 0.00) for 1H NMR spectra measured in C6D6OS. This solvent was also used for 13C NMR spectra.

#### 2.2 Materials

Black rice bran was obtained from Real Co., Ltd. 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide), 4-nitroquinolinol 1-oxide (4NQO), N-methyl-N’-nitro-N-nitrosoguanidine (MNNG), aflatoxin B1 (Afb1), 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and veratric acid were purchased from Wako Pure Chemical Co. S9 (supernatant of 9000g) and coenzyme NADPH, NADH, and G-6-P were purchased from Oriental Yeast Co.

#### 2.3 *Umu* Test

The *umu* test for detecting the chemicals-induced SOS response was carried out according to the method of Oda *et al.* (2) using *Salmonella typhimurium* TA1535/pSK1002, the pSK1002 plasmid of which carries an *umuC*-*lacZ* fused gene. The overnight culture of bacterial strain was diluted 50-fold into TGA medium (1% Bactotryptone, 0.5% NaCl, and 0.2% glucose; supplemented with 20 mg/L ampicillin) and incubated at 37°C until the bacterial density reached 0.25-0.30 in OD660. The bacterial culture was subdivided into 2.1 mL portions in test tubes, and the test compound (50 µL), 0.1 M phosphate buffer (300 µL, pH 7.4), and mutagens furylfuramide (50 µL, 2 µg/mL in DMSO), 4NQO (50 µL, 20 µg/mL in DMSO) and MNNG (50
μL, 200 μg/mL in DMSO) were added to each tube. In the case of AfB1 (50 μL, 20 μg/mL in DMSO) and Trp-P-1 (50 μL, 40 μg/mL in DMSO), 300 μL of S9-metabolizing enzyme mixture including the cofactors was added instead of the phosphate buffer. As a positive control an equivalent volume of DMSO was added instead of the test compound, whereas with negative control an equivalent volume of DMSO was added instead of both the test compound and the mutagen. After 2 h of incubation at 37°C with shaking, the culture was centrifuged (3000 rpm) to collect cells, which were centrifuged in 2.5 mL of PBS. The level of β-galactosidase activity was calculated according to the method of Miller’s method (23). Fractions (0.25 mL) of the culture were diluted with 2.25 mL of Z buffer, and 0.1% SDS solution (50 μL) and chloroform (10 μL) were added to each fraction. The enzyme reaction was initiated by the addition of 0.25 mL of 2-nitrophenyl-β-D-galactopyranoside solution (ONPG; 4 mg/mL in 0.1M phosphate buffer, pH 7.4) at 28°C. After 15 min, the reaction was stopped by addition of 0.1 M Na2CO3, and the absorbance at OD620 and OD590 was measured. Using the remainder of culture, the bacterial density was measured at OD600. The unit of β-galactosidase activity was calculated according to the method of Miller (23).

2.4 Preparation of Activated Trp-P-1
Preparation of activated Trp-P-1 was carried out according to the method of Arimoto et al. (24).

2.5 UV Irradiation
The overnight culture of the tester bacterial strain (S. typhimurium TA1535/pSK1002) incubated at 37°C in Luria broth was diluted 50-fold with fresh TGA medium and incubated at 37°C until the optical density at 600 nm of 0.25-0.30 was reached. The cultures were then collected by centrifugation and suspended in 5 mL of 0.1 M phosphate buffer. The cell suspensions were then poured into Petri dishes and exposed to UV light (2.0 J/m²) for 10 s using a germicidal lamp at room temperature.

2.6 Purification and Identification of the Suppressive Compounds
As shown in Fig. 1, black rice bran (1.0 kg) was refluxed with methanol for 9 h to give a methanol extract (186.0 g). This extract was suspended in water and re-extracted with hexane, chloroform, ethylacetate, butanol and water, respectively. Each soluble fraction was concentrated under reduced pressure to give hexane (54.5 g), chloroform (13.8 g), ethylacetate (5.3 g), butanol (21.6 g) and water (90.8 g) fractions. To purify the compound responsible for suppression of the SOS-inducing activity, these fractions were evaluated with the umu test. The ethylacetate fraction showed a suppressive effect. The ethylacetate fraction was fractionated to fractions 1 and 2 by silica gel column chromatography with chloroform and acetone as eluents. Fraction 1 showed a suppression of the furylfuramide-induced SOS response in the umu test, and this fraction was partitioned with 5% NaHCO3 solution. The aqueous layer was acidified with diluted HCl and then extracted with ethylacetate to yield the acidic fraction 4 (2.4 g). The acidic fraction 4 showed a suppression of the SOS-inducing activity on furylfuramide in the umu test. This fraction was repeatedly fractionated by silica gel column chromatography using the umu test as a guide. Finally, suppressive compounds 1 (291 mg) and 2 (381 mg) were isolated from black rice bran. Compounds 1 and 2 were identified as vanillic acid (1) and protocatechuc acid (2) by GC, GC/MS, IR and 1H and 13C NMR, respectively (Fig. 2).

2.7 Compound 1
Compound 1 was a brownish white powder: MS, m/z 168 (M+, 100%), 153 (74.3%), 125 (16.7%), 97 (34.4%), 79 (8.6%), 53 (6.9%), 51 (14.6%); IR vmax KBr (cm−1) 3427.9, 1674.9, 1598.7, 1241.0, 1030.0; 1H NMR (C2D6OS) δ 3.82 (3H, s, OCH3), 6.85 (1H, d, J = 2, 9, H-5), 7.45 (1H, dd, J = 2, 9, H-6), 7.35 (1H, d, J = 2, H-2); 13C NMR (C2D6OS) δ 167.28 (COOH), 150.03 (C-4), 131.29 (C-3), 123.49 (C-2), 121.82 (C-1), 116.76 (C-6), 115.10 (C-5), 113.23 (C-2), 55.77 (OCH3). Compound 1 was identified as vanillic acid [4-hydroxy-3-methoxy benzoic acid] from these spectral data.

2.8 Compound 2
Compound 2 was a yellowish white powder: MS, m/z 154 (92.4%), 137 (M+, 100%), 109 (29.2%), 97 (5.6%), 81 (14.6%), 63 (15.7%), 53 (20.6%); IR vmax KBr (cm−1) 3266.8, 1667.2, 1600.6, 1317.1, 1267.0, 765.6; 1H NMR (C2D6OS) δ 6.80 (1H, d, J = 8.5, H-5), 7.30 (1H, dd, J = 2, 8.5, H-6), 7.35 (1H, d, J = 2, H-2); 13C NMR (C2D6OS) δ 167.28 (COOH), 150.03 (C-4), 144.94 (C-3), 121.97 (C-1), 121.93 (C-6), 116.76 (C-2),
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Compound 2 was identified as protocatechuic acid [3,4-dihydroxy benzoic acid] from these spectral data.

2.9 Methyl Esters of Compounds 1, 2 and 3 (1Me, 2Me and 3Me)

Methyl esters of 1, 2 and veratric acid [3,4-dimethoxy benzoic acid] (3) were obtained by reaction with diazomethane. These structures were identified by GC, GC/MS and IR (Fig. 2).

2.10 Methyl Ester 1Me

MS, m/z 182 (56.9%), 153 (8.9%), 151 (M⁺, 100%), 123 (16.0%), 107 (36.5%), 108 (9.3%), 52 (13.9%); IR νmax KBr (cm⁻¹) 3404.7, 2952.5, 2840.6, 1713.4, 1597.7. Methyl ester 1Me was identified as methyl vanillate [4-
hydroxy-3-methoxy methyl benzoate] from these spectral data.

2-11  Methyl Ester 2Me
MS, m/z 168 (41.8%), 137 (M+, 100%), 123 (1.0%), 109 (21.0%), 91 (1.0%), 81 (11.0%), 51 (18.0%); IR νmax KBr (cm⁻¹) 3415.3, 2955.4, 2839.7, 1694.2, 1605.5. Methyl ester 2Me was identified as methyl protocatechuic acid (3,4-dihydroxy methyl benzoate) from these spectral data.

2-12  Methyl Ester 3Me
MS, m/z 197 (10.0%), 196 (M+, 100%), 165 (98.5%), 137 (9.7%), 125 (98.5%), 79 (30.0%), 51 (21.5%); IR νmax KBr (cm⁻¹) 2952.5, 2839.7, 1712.5, 1600.6, 1271.8. Methyl ester 3Me was identified as methyl veratract [3,4-dimethoxy methyl benzoate] from these spectral data.

3 Results
3-1  Fractionation of the Extract from Black Rice Bran and Isolation of Suppressive Compounds 1 and 2
The methanol extract of black rice bran was fractionated to search for suppressive compounds using the umu test as a guide (Fig. 1). 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 acidic fraction (fraction 4) of the ethylacetate fraction exhibited a suppressive effect of the furylfuramide-induced SOS response in S. typhimurium TA1535/pSK1002. After this fraction was fractionated, the suppressive fraction 6 eluted with 85:15 and fraction 7 eluted with 80:20 hexane/ethylacetate as eluents had clear-cut dose-response effects in the fractionation (fractions 5-8). Finally, suppressive compound 1 was isolated from the suppressing fraction 6, and suppressive compound 2 was isolated from the suppressing fraction 7. Compounds 1 and 2 were identified as vanillic acid (1) and protocatechuic acid (2) by GC, GC/MS, IR and ¹H and ¹³C NMR, respectively (Fig. 2).

3-2  Inhibition of SOS-Inducing Activity by Compounds 1 and 2
The suppressive effects of compounds 1 and 2 were evaluated in the umu test. Compounds 1 and 2 exhibited inhibitions on the furylfuramide-induced SOS response (Table 2). Compounds 1 and 2 suppressed 37.7 and 44.5%, respectively, of the SOS-inducing activity on furylfuramide at a concentration of 1.20 μmol/mL (Fig. 3). Compounds 1 and 2 were also assayed with other mutagens, 4NQO and MNNG, which do not require a liver-metabolizing enzymes mixture (Table 2). Compounds 1 and 2 suppressed 36.6 and 48.3% of the SOS-inducing activity on 4NQO at a concentration of 1.20 μmol/mL (Fig. 4). Compounds 1 and 2 showed suppressive effects (25.6 and 36.7%) at a concentration of 1.20 μmol/mL of the SOS-inducing activity on MNNG (Fig. 4). These compounds were also assayed with AfB₁ and Trp-P-1, which require liver metabolic activation (Table 3). As shown in Fig. 5, compounds 1 and 2 suppressed 52.3 and 69.5% of the SOS-inducing activity on AfB₁ at a concentration of 1.20 μmol/mL, and the ID₅₀ (50% inhibitory dose) values of 1 and 2 were 1.10 and 0.92 μmol/mL. Compounds 1 and 2 suppressed 23.3 and 54.0% of the SOS-inducing activity on MNNG at a concentration of 1.20 μmol/mL. The ID₅₀ value of 2 was 1.11 μmol/mL (Fig. 5). As these results of the umu test, the suppressive effects of 1 and 2 on 4NQO are similar to the suppressive effects observed in the case of furylfuramide,
Suppressive Effect of Methyl Esters of Compounds 1 and 2 (1Me and 2Me)

Methyl esters of 1 and 2 (1Me and 2Me) were examined for their ability to suppress the SOS-inducing activity on furylfuramide, 4NQO and MNNG (Table 2). As shown in Fig. 3, methyl esters 1Me and 2Me suppressed 41.4 and 49.2% of the SOS-inducing activity on furylfuramide at a concentration of 1.20 μmol/mL. Methyl esters 1Me and 2Me showed suppressive effects (41.4 and 54.9% at a concentration of 1.20 μmol/mL) of the SOS-inducing activity on 4NQO, and the ID₅₀ value of 2Me was 1.07 μmol/mL (Fig. 4). Methyl esters 1Me and 2Me suppressed 30.0 and 44.4% of the SOS-inducing activity on MNNG at a concentration of 1.20 μmol/mL (Fig. 4). These methyl esters 1Me and 2Me were also assayed with AfB₁ and Trp-P-1 (Table 3). As shown in Fig. 5, methyl esters 1Me and 2Me suppressed 86.2 and 90.8% of the SOS-inducing activity on AfB₁ at a concentration of 1.20 μmol/mL, and the

Table 2  Suppressive Effects of Compounds 1-3 and 1Me-3Me on Furylfuramide a, 4NQO b and MNNG c Using S. typhimurium TA1535/pSK1002.

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a Furylfuramide (2 μg/mL in DMSO) was added at 50 μL. b 4NQO (20 μg/mL in DMSO) was added at 50 μL. c MNNG (200 μg/mL in DMSO) was added at 50 μL. d β-galactosidase activity (units).
Table 3  Suppressive Effects of Compounds 1-3 and 1Me-3Me on AfB₁⁠²⁰, Trp-P-1⁠²³ and Activated Trp-P-1⁠²⁴ Using *S. typhimurium* TA1535/pSK1002.

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| 1        | 711  | 211     | 211               | 595     | 211                     |
| 2        | 711  | 211     | 211               | 441     | 211                     |
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| 1Me      | 711  | 211     | 211               | 328     | 211                     |
| 2Me      | 711  | 211     | 211               | 266     | 211                     |
| 3Me      | 711  | 211     | 211               | 356     | 211                     |

| 1        | 766  | 146     | 146               | 695     | 146                     |
| 2        | 766  | 146     | 146               | 583     | 146                     |
| 3        | 766  | 146     | 146               | 716     | 146                     |
| 1Me      | 766  | 146     | 146               | 614     | 146                     |
| 2Me      | 766  | 146     | 146               | 536     | 146                     |
| 3Me      | 766  | 146     | 146               | 671     | 146                     |

ᵃ AfB₁ (20 μg/mL in DMSO) was added at 50 μL. ᵇ Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL. ᶜ Activated Trp-P-1 was added at 50 μL. ᵈ β-galactosidase activity (units).
ID₅₀ values of 1Me and 2Me were 0.66 and 0.49 μmol/mL. Methyl esters 1Me and 2Me showed suppressive effects (76.7 and 89.0% at a concentration of 1.20 μmol/mL) of the SOS-inducing activity on Trp-P-1, and the ID₅₀ values of 1Me and 2Me were 0.83 and 0.62 μmol/mL (Fig. 5). As these results of the umu test, methyl esters 1Me and 2Me had greater suppressive effects of the SOS genes against all mutagens than 1 and 2.

### 3.4 Inhibition of SOS-Inducing Activity by Compound 3 and Methyl Ester of Compound 3 (3Me)

For confirming the structure-activity relationship, veratric acid (3,4-dimethoxy benzoic acid) (3) and methyl ester of 3 (3Me) were obtained. Compounds 3 and 3Me were also examined for their ability to suppress the SOS-inducing activity on furylfuramide, 4NQO and MNNG (Table 2). Compounds 3 and 3Me showed suppressive effects (29.4 and 33.9% at a concentration of 1.20 μmol/mL) of the SOS-inducing activity on furylfuramide (Fig. 3). Compounds 3 and 3Me suppressed 28.8 and 34.1% of the SOS-inducing activity on 4NQO at a concentration of 1.20 μmol/mL (Fig. 4). Compounds 3 and 3Me showed suppressive effects (43.1 and 52.2% at a concentration of 1.20 μmol/mL) of the SOS-inducing activity on MNNG, and the ID₅₀ value of 3Me was 1.16 μmol/mL (Fig. 4). Compounds 3 and 3Me were also assayed with AfB₁ and Trp-P-1 (Table 3). Compounds 3 and 3Me suppressed 40.1 and 78.4% of the SOS-inducing activity on AfB₁ at a concentration of 1.20 μmol/mL, and the ID₅₀ value of 3Me was 0.82 μmol/mL (Fig. 5). As shown in Fig. 4, compounds 3 and 3Me suppressed 20.9 and 71.0% of the SOS-inducing activity on Trp-P-1 at a concentration of 1.20 μmol/mL, and the ID₅₀ value of 3Me was 0.91 μmol/mL. As these results of the umu test, compound 3 had weaker suppressive effects of the SOS genes against furylfuramide, 4NQO, AfB₁, Trp-P-1 than 1 and 3Me. Methyl ester 3Me also had weaker suppressive effects of the SOS genes against furylfuramide, 4NQO, AfB₁, Trp-P-1 than 1Me. However, compounds 3 and 3Me had stronger suppressive effects of the SOS genes against MNNG than 1 and 1Me. Compound 3Me had greater suppressive effects of the SOS genes against all chemical mutagens than 3.

### 3.5 Suppressive Effects of Compounds 1-3 and 1Me-3Me on Metabolic Activation of Trp-P-1

The suppressive effects of compounds 1-3 and 1Me-3Me on metabolic activation of Trp-P-1 were determined by using the umu test. The value of β-galactosidase activity observed in the absence of these compounds was for activated Trp-P-1. As shown in Table 3 and Fig. 5, compounds 1-3 suppressed 11.5, 29.6 and 8.2% of the SOS-inducing activity on activated Trp-P-1 at a concentration of 1.20 μmol/mL. As shown in
Table 3 and Fig. 4, methyl esters 1Me-3Me suppressed 24.6, 37.1 and 15.4% of the SOS-inducing activity on activated Trp-P-1 at a concentration of 1.20 \( \mu \)mol/mL. Suppressive effects of 1-3 and 1Me-3Me on activated Trp-P-1 were decreased compared with those on Trp-P-1.

### 3.6 Suppressive Effects of Compounds 1-3 and 1Me-3Me on UV Irradiation

The suppressive effects of compounds 1-3 and 1Me-3Me on UV irradiation-induced SOS response were evaluated in the test using *S. typhimurium* TA1535/pSK1002. Compounds 1-3 and 1Me-3Me did not show suppressive effects on UV irradiation (data not shown).

### 4 Discussion

The suppressive compounds of SOS-inducing activity in black rice bran were identified as due to vanillic acid (1) and protocatechuic acid (2). These compounds showed suppressive effects on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against furylfuramide, 4NQO and MNNG, which do not require liver-metabolizing enzymes, and AFB_{1} and Trp-P-1, which require liver-metabolizing enzymes. As shown in Tables 2 and 3 and Figs. 3-5, compound 1 had weaker suppressive potencies against furylfuramide, 4NQO, MNNG, AFB_{1} and Trp-P-1 than 1Me, and compound 2 also had weaker suppressive potencies against all these chemical mutagens than 2Me. The difference in structure between 1 and 1Me and between 2 and 2Me is a methyl-esterification of carboxyl group. These results indicated that a methyl-esterification of carboxyl group is an important factor for suppressing the SOS-inducing activity on all chemical mutagens. As shown in Tables 2 and 3 and Figs. 3-5, compound 2 had greater suppressive potency against all chemical mutagens than 1. The difference in structure between 1 and 2 is a hydroxyl group at the C-3 position. These results indicated that a hydroxyl group at the C-4 position is also an important factor for suppressing the SOS-inducing activity on furylfuramide, 4NQO, AFB_{1} and Trp-P-1.

These compounds were examined for the ability to suppress the metabolic activation of Trp-P-1 by S9. As shown in Table 3 and Fig. 5, these compounds suppressed the weaker SOS induction on activated Trp-P-1 than they did on Trp-P-1. These results suggested the possibility that the inhibition of the SOS-inducing activity on Trp-P-1, which was caused by compounds 1-3 and 1Me-3Me was due to the inhibition of metabolic activation by S9.

For mutagenic activation of furylfuramide (*cis* form), *cis-trans* isomerization (25-27) and reduction of the nitro group of 5-nitrofuran (28,29) are important in the metabolic pathway. The *cis-trans* isomerization is based on the formation of nitro anion radicals. *cis*-Furylfuramide receives a single electron derived from an enzyme system to form the anion radical. Spin density on the olefinic double bond results in free rotation between the olefinic carbons followed by conversion to its thermodynamically more stable trans isomer. The nitro group of 5-nitrofuran is activated by the reductive metabolism associated with nitroreductases in bacteria. The main pathway for nitrofuran activation would be via reduction to a hydroxylamine intermediate, which could react with DNA though a nitrenium ion. An alternative reactive intermediate, the ring-opened acrylonitrile derivative, could form through rearrangement of the hydroxylamine intermediate. It has been shown that the acrylonitrile derivative readily forms conjugates with glutathione, nercaptoethanol, and thiol groups of proteins. These conjugates increase the mutation frequency in *S. typhimurium* TA100, suggesting that the acrylonitryle derivative is also capable of interacting with DNA. Vanillic acid and protocatechuic acid exhibited inhibitions of SOS induction on furylfuramide. Therefore, vanillic acid and protocatechuic acid may block these reactive metabolic pathways and/or activation of nitro anion radicals on furylfuramide.

With respect to mutagenic activation of 4NQO, metabolic activation of 4NQO by a nitro reduction enzyme to 4-hydroxyaminoquinoline 1-oxide (4HAQO) (30,31), which is believed to be a proximate derivative of the potent carcinogen 4NQO, reaction of 4HAQO with DNA to yield three primary adducts (N2-guanine, C8-guanine, and N6-adenine adducts) (32), and access of 4NQO and/or 4HAQO to the target cell of bacteria.
are necessary. Vanillic acid and protocatechuic acid exhibited inhibitions of SOS induction on 4NQO. Therefore, vanillic acid and protocatechuic acid may block this reactive metabolic activation of 4NQO, which is reacted with a nitro reduction enzyme in cells of bacteria.

Blocking effects of vanillic acid and protocatechuic acid may be caused by the possible involvement of their antioxidant and scavenging properties. Vanillic acid and protocatechuic acid had antioxidant activities and radical scavenging (13). Therefore, the blocks by vanillic acid and protocatechuic acid could arise from their scavenging ability to trap hydroxyl radicals originating from metabolites of furylfuramide and 4NQO and/or activation of nitro anion radicals on furylfuramide with a hydroxy group.

Vanillic acid and protocatechuic acid and their methyl esters had weak suppressive effects of the SOS-inducing activity on the direct alkylating agent MNNG. On the other hand, veratric acid and its methyl ester had great suppressive effects of SOS-inducing activity on the direct alkylating agent MNNG. It was found that veratric acid and its methyl ester were operative to inhibit the SOS-inducing activity on the direct alkylating agent MNNG. As shown in Table 2 and Fig. 4, compound 3 had a greater suppressive potency against the direct alkylating agent MNNG than 1. As shown in Table 2 and Fig. 4, compound 3Me had a greater suppressive potency against the direct alkylating agent MNNG than 1Me. The difference in structure between 3 and 1 and between 3Me and 1Me is a methoxy group at C-4 position. These results indicated that a methoxy group at C-4 position is an important factor for suppressing the SOS-inducing activity of the direct alkylating agent MNNG.

Benzoic acids as vanillic acid and veratric acid did not show any bioantimutagenic activity on 4NQO in Escherichia coli WP2s uvrA type (33). On the other hand, in the umu test, vanillic acid and veratric acid showed suppressive effects on 4NQO. This illustrates the discrepancy between the results of the umu test and this test. We think this discrepancy is probably due to the difference of mechanisms of the suppressive effects. Protocatechuic acid has bio-antischistogenic activity in CHO cells and mice (34). However, inhibition of mutagen-induced SOS response by protocatechuic acid and vanillic acid has not been reported.

In summary, this research suggests that suppressive compounds on SOS response against mutagens in black rice bran were primarily vanillic acid (1) and protocatechuic acid (2). Compounds 1 and 2 showed potent suppressive effects of SOS-inducing activity by chemical mutagens, and the characteristic activity of the respective compounds was dependent upon the hydroxyl groups at the C-3 and C-4 position.

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