Antimutagenic Activity of Falcarindiol from *Peucedanum praeruptorum*

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A methanol extract from *Peucedanum praeruptorum* 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 methanol extract from *P. praeruptorum* was re-extracted with hexane, dichloromethane, *n*-butanol, and water, respectively. A suppressive compound in the hexane extract fraction was isolated by SiO_2 column chromatography and identified as falcarindiol by EI-MS, IR, and ^1H and ^{13}C NMR spectroscopy. Falcarindiol exhibited an inhibition of the SOS-inducing activity of furylfuramide in the *umu* test. Gene expression was suppressed 75% at less than 0.15 μmol/mL, and the ID_{50} value was 0.10 μmol/mL. The diacetate compound of falcarindiol did not show any suppressive effect on the SOS induction of furylfuramide. Falcarindiol 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. The falcarindiol ID_{50} value versus Trp-P-1 was 0.096 μmol/mL. The antimutagenic activities of falcarindiol and falcarindiol diacetate against furylfuramide and Trp-P-1 were tested by an Ames test using *S. typhimurium* TA100, which indicated that falcarindiol suppressed the mutagenicity of furylfuramide and Trp-P-1 and falcarindiol diacetate suppressed the mutagenicity of Trp-P-1.

Keywords: *Umbelliferae; Peucedanum praeruptorum; falcarindiol; antimutagenic activity; umu test; Ames test*

INTRODUCTION

Umbellifers are recognized as popular natural aromatic and important medicinal plants of traditional Chinese medicines. They produce essential oils, terpenoids, saponins, aromatic compounds, flavonoids, chromones, and coumarins (Okuyama et al., 1991). The material used for the present investigation was identified as Bai-Hua Quan Hu (Byakka Zenko in Japanese), the root of *Peucedanum praeruptorum* Dunn. Qian-Hu, the roots of *P. praeruptorum*, is a traditional Chinese medicine used in alimentary and bronchial disorders and chest pains (Kozawa et al., 1981). Coumarins are widely distributed in plants (Härmälä et al., 1992). *P. praeruptorum* is known to be a rich source of seselin-type coumarins (Sasakibara et al., 1984). So far, there are no reports of acetylene compounds from *P. praeruptorum*. Polyacetylenes have been found in many plants, usually as several different compounds and then only in very small amounts (Muir et al., 1982). Acetylenic compounds have often been shown to be toxic to bacteria, nematodes, and mammals (Kemp et al., 1978). The polyacetylenic compound falcarindiol is known to have antifungal activity as well as cytotoxic activity (Kemp, 1978; Cunsolo et al., 1993).

In the evaluation of the carcinogenicity or mutagenicity of environment 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., 1995a), we found that the methanol extract of *P. praeruptorum* 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 *P. praeruptorum*.

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 Fourier transform infrared 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

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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 *P. praeruptorum* was obtained from Takasago Yakugiyo Co. (Osaka). Furylfuramide was one of the nitrofran derivatives and had been widely used as a food preservative in Japan. Its genetic effects were reported by many workers, and this compound is one of the well-known mutagens (Kada, 1973; Tonomura et al., 1973). Heterocyclic amine from protein pyrolysis products were highly mutagenic components when the liver S9 fraction was used as the activating system (Kosuge et al., 1978; Kato et al., 1983). Trp-P-1 is one of the heterocyclic aromatic amines. Furylfuramide and Trp-P-1 were purchased from Wako Pure Chemical Co. S9 fractions prepared from livers of male rats pretreated with phenobarbital and 5,6-benzoflavone were purchased from Oriental Yeast Co. The cofactor for the S9 mix was prepared by the detailed method of Yahagi et al. (1977).

***umu* Test.** The *umu* test is based upon the abilities of carcinogens and mutagens to introduce expression of an *umu* gene in *Salmonella typhimurium* TA1535/pSK1002 (Oda et al., 1985), in which a plasmid (pSK1002) carrying a fused gene (*umuC′-′lacZ*) had been introduced. The SOS-inducing potency is estimated by the measurement of the level of *umu* operon expression in terms of cellular β-galactosidase activity. The SOS responses appear after DNA damage or interference with DNA replication (Little et al., 1982; Walker, 1984).

Briefly, an overnight culture of the tester bacterial strain in Luria broth (1% Bactotryptone, 0.5% NaCl, and 0.5% yeast extract) was diluted 50-fold with fresh 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 at 600 nm reached 0.25−0.30. The culture was divided into 2.3 mL portions in test tubes. The test compound (50 μL, diluted in DMSO), 0.1 M phosphate buffer (300 μL, pH 7.4), and furylfuramide (50 μL, 1 μg/mL in DMSO) were added to each tube. In the case of Trp-P-1 (50 μL, 40 μg/mL in DMSO), 300 μL of S9-metabolizing enzyme instead of 0.1 M phosphate buffer mixture was added. 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 cell density was read at 600 nm with one portion (1.0 mL) of the suspension. Using the other portion (0.25 mL), the level of β-galactosidase activity in the cell was assayed by the method of Miller (1972).

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). The test compound (50 μL), 0.1 M phosphate buffer (500 μL), and furylfuramide (50 μL, 0.2 μg/mL in DMSO) were added to each test tube. In the case of Trp-P-1 (50 μL, 40 μg/mL in DMSO), 500 μL of S9-metabolizing enzyme mixture instead of 0.1 M phosphate buffer was added. The culture of the tester bacterial strain (*S. typhimurium* TA100) was divided into 100 μL portions into the test tube. The mixture was preincubationed at 37 °C for 20 min, mixed with 2.0 mL of top agar at 45 °C, and poured onto a minimal-glucose agar plate. After incubation for 2 days at 37 °C, the colonies on the plate were counted.

Purification and Identification of the Suppressive Compound 1. The dry powder (2.5 kg) of *P. praeruptorum* was refluxed with methanol for 12 h and concentrated under reduced pressure to give the methanol extract (485 g). This extract was suspended in water (1 L) and partitioned between hexane (500 mL) and water, dichloromethane (1 L) and water, and finally *n*-butanol (1 L) and water successively. Each soluble fraction was concentrated under reduced pressure to give hexane (111 g), dichloromethane (138 g), *n*-butanol (68 g), and water (177 g), respectively. To pursue the compound responsible for the suppression of the SOS-inducing activity, these fractions were tested. The elution solvent for each fraction and fraction volumes are given in Figure 1. As shown in Figure 1, the hexane fraction had positive activity, whereas the dichloromethane, *n*-butanol, and water fractions did not show any activity. Therefore, the hexane-soluble materials were separated into fractions 1−5 by SiO_2 column chromatography (13 × 50 cm and 3 kg of silica gel (200 mesh)) with hexane, diethyl ether, ethyl acetate, and methanol as eluents. Fraction 2 showed suppression of the SOS-inducing activity of furylfuramide in the *umu* test. Therefore, the material in fraction 2 was further separated into fractions 6−10 by SiO_2 column chromatography (8 × 30 cm and 300 g (300 mesh)), and then fraction 8 was repeatedly fractionated by SiO_2 column chromatography (5 × 20 cm, 150 g of silica gel (300 mesh) and solvent system of hexane and diethyl ether). Finally, suppressive compound **1** (240 mg) was isolated and stored at −20 °C in the dark.

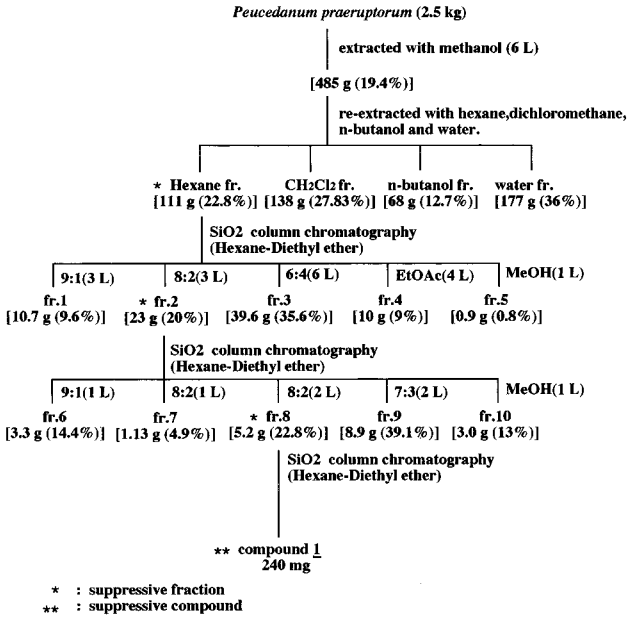


Figure 1. Isolation scheme for the suppressive compound from *P. praeruptorum*.

Suppressive Compound 1. Compound **1** was a colorless oil: $[\alpha]^{20}_D$ +126° (*c* 1.0, $CHCl_3$); EI-MS *m*/*z* 260 (M^+); ^1H NMR, ^{13}C NMR and IR properties were identical with those of falcarindiol (Kozawa et al., 1983; Baba et al., 1987). The suppressive compound **1** was identified as falcarindiol (1,9-heptadecadiene-4,6-diyne-3,8-diol) from these spectral data and physical properties.

Acetylation of Suppressive Compound 1 (1-Ac). Compound **1**-Ac was obtained by reaction of **1** with acetic anhydride using pyridine as a catalyst. The compound was a colorless oil. IR, ^1H NMR and ^{13}C NMR properties were identical with those of falcarindiol diacetate (Kozawa et al., 1983; Kohda et al., 1983). Compound **1**-Ac was identified as falcarindiol diacetate from these spectral data.

RESULTS

Fractionation of the Extract of *P. praeruptorum* and Isolation of Falcarindiol (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 200, 100, and 40 μg/mL. As shown in Figure 1 and Table 1, the hexane fraction from *P. praeruptorum* showed a suppressive effect on *umu* gene expression of the SOS responses in *S. typhimurium* TA1535/pSK1002 against furylfuramide. The dichloromethane, *n*-butanol, and water fractions did not show this suppressive effect. After fractionating the hexane fraction, 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 8 had the effect of suppressing the SOS-inducing activity. The suppressive

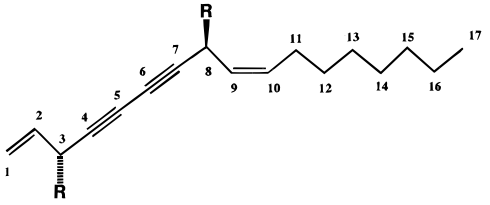
Table 1. Suppressive Effect of *P. praeruptorum* Fractions on Furylfuramide[a] Using *S. typhimurium* TA1535/pSK1002

		dose response[c]			
sample	control[b]	200	100	40	0 (μg/mL)
MeOH extract	240.6	540.0	840.0	915.2	1010.0
hexane fraction	261.6	430.0	530.2	780.3	955.0
CH_2Cl_2 fraction	261.6	770.3	740.2	860.5	955.0
n-BuOH fraction	261.6	903.0	883.0	824.0	955.0
water fraction	261.6	921.0	928.0	923.0	955.0
fraction 1	114.4	640.3	705.8	781.2	743.0
fraction 2	114.4	290.2	393.0	656.9	743.0
fraction 3	114.4	550.3	620.6	721.7	743.0
fraction 4	114.4	589.7	660.0	825.2	743.0
fraction 5	114.4	710.6	760.0	800.8	743.0
fraction 6	224.5	639.2	601.2	662.3	540.4
fraction 7	224.5	484.2	570.3	699.2	540.4
fraction 8	224.5	300.4	350.2	470.2	540.4
fraction 9	224.5	460.5	480.5	620.7	540.4
fraction 10	224.5	520.5	530.5	570.6	540.4

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

fraction 8 was fractionated by repeated SiO_2 column chromatography to yield compound **1.**

Structure Determination of Falcarindiol (1) and Falcarindiol Diacetate (1-Ac). Compound **1** gave a molecular ion as a base peak in the EI mass spectrum at *m/z* 260. The IR spectrum of **1** showed characteristic absorption bands caused by acetylene bonds at 2232 and 2147 cm^{-1}. The ^{13}C NMR spectrum of **1** showed the signals which can be attributed to acetylenic bonds at 79.9 (C-4), 68.7 (C-5), 70.2 (C-6), and 78.3 (C-7), respectively. The ^1H NMR spectrum of **1** confirmed the presence of a methyl group at δ 0.88. The signals at H-2, H-1a, and H-1b appeared at δ 5.94, 5.48, and 5.26, respectively. The signals at H-9 and H-10 appeared at δ 5.52 and 5.61, respectively, confirming the presence of olefinic protons. The signals at H-3 and H-8 appeared at δ 4.94 and 5.22, respectively, indicating the presence of the methine proton attached to the olefin group and acetylene group. Thus compound **1** was identified as falcarindiol. Compound **1**-Ac showed a molecular ion



1 : R = OH
1-Ac : R = OAc

at *m/z* 344 in the EI mass spectrum. The ^{13}C NMR spectra of compound **1**-Ac showed signals appearing at 64.36 (C-3) and 60.02 (C-8), respectively, confirming the presence of acetyl groups. The ^1H NMR spectra indicated the appearence of signals at δ 2.07 and δ 2.10 (acetyl protons), which further confirmed the structure of **1**-Ac as falcarindiol diacetate.

Inhibition of the SOS-Inducing Activity of Falcarindiol (1) and Falcarindiol Diacetate (1-Ac). The suppressive effect of compounds **1** and **1**-Ac was determined in the *umu* test. As shown in Table 2, **1** exhibited inhibition of the SOS induction by furylfuramide, whereas **1**-Ac did not. Compound **1** completely suppressed SOS induction at 0.15 μmol/mL, and the ID_{50} value was 0.10 μmol/mL. In addition, **1** and **1**-Ac were assayed with another mutagen (Trp-P-1), which requires

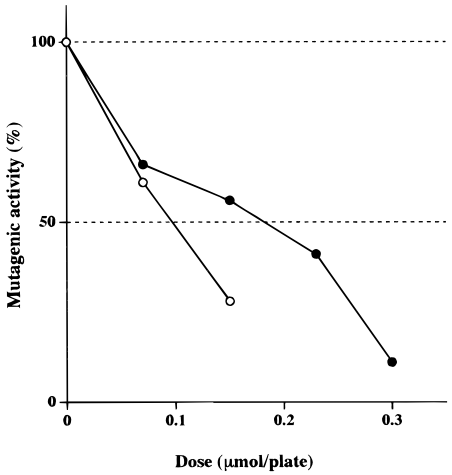


Figure 2. Effect of falcarindiol on the mutagenicity of furylfuramide and Trp-P-1 in *S. typhimurium* TA100: (○) effect of falcarindiol on the mutagenicity of furylfuramide; (●) effect of falcarindiol on the mutagenicity of Trp-P-1. Furylfuramide (1 μg/mL in DMSO) was added at 50 μL/plate. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL/plate. In the case of furylfuramide, falcarindiol was toxic at <0.23 μmol/plate.

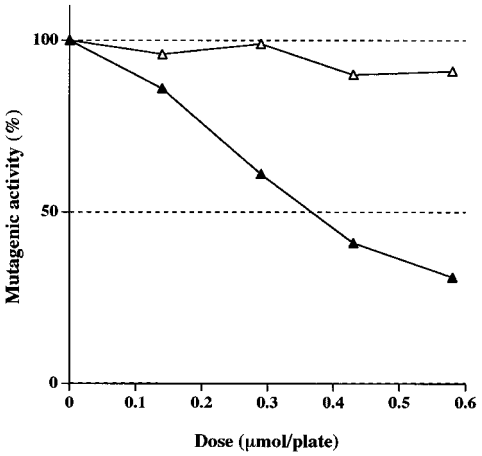


Figure 3. Effect of falcarindiol diacetate on the mutagenicity of furylfuramide and Trp-P-1 in *S. typhimurium* TA100: (△) effect of falcarindiol diacetate on the mutagenicity of furylfuramide; (▲) effect of falcarindiol diacetate on the mutagenicity of Trp-P-1. Furylfuramide (0.5 μg/mL in DMSO) was added at 20 μL/plate. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL/plate.

liver-metabolizing enzymes for mutagenicity. In this case, **1** showed a suppressive effect similar to that with furylfuramide, whereas **1**-Ac did not. The ID_{50} value of **1** against Trp-P-1 was 0.096 μmol/mL.

Antimutagenic Activity of Falcarindiol (1) and Falcarindiol Diacetate (1-Ac). 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, **1** suppressed 72% of the mutagenicity of furylfuramide at 0.15 μmol/plate, and the ID_{50} value was 0.095 μmol/plate. In the case of furylfuramide, **1** showed toxicity at a concentration of 0.23 μmol/plate. Similarly, **1** suppressed the mutagenicity of Trp-P-1 completely at 0.30 μmol/plate, and the ID_{50} value was 0.18 μmol/plate. Also, as shown in Figure 3, **1**-Ac did not suppress the mutagenicity of furylfuramide. However, compound **1**-Ac suppressed 69% of the mutagenicity of Trp-P-1 at 0.58 μmol/plate, and the ID_{50} value was 0.37 μmol/plate. From the result of the *umu* test, **1**-Ac did not suppress

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

				dose response[c]			
chemical	furylfuramide	Trp-P-1	control	0.15	0.12	0.06	0.03 (μmol/mL)
falcarindiol	546.3		172.2	263.0	321.8	466.7	493.9
falcarindiol diacetate	546.3		172.2	467.7	490.2	505.1	527.6
falcarindiol		254.4	144.0	136.0	181.2	223.4	235.6
falcarindiol diacetate		254.4	144.0	226.8	234.5	240.0	246.6

[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).

the mutagenicity of furylfuramide and Trp-P-1, but it did suppress the mutagenicity of Trp-P-1 in the Ames test.

DISCUSSION

The antimutagenic compound in *P. praeruptorum* was clearly identified as falcarindiol. This compound had a suppressive effect on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against furylfuramide and Trp-P-1, which requires liver-metabolizing enzymes. From the results of the *umu* test, **1** showed suppressive effects on the SOS-inducing activity of furylfuramide and Trp-P-1. On the other hand **1**-Ac did not show suppressive effects of both mutagens. This result suggests that two hydroxyl groups are one of the major factors for the suppression of the SOS-inducing activity of both mutagens. In the case of the Ames test, **1** suppressed the mutagenicity of furylfuramide and Trp-P-1 as well as the *umu* test. Although **1**-Ac did not suppress the mutagenicity of furylfuramide similar to the *umu* test, it suppressed the mutagenicity of Trp-P-1. The difference between the result of the Ames and the *umu* tests may be caused by the difference of these methods (Miyazawa et al., 1995b). The principle of the *umu* test is based on the ability of chemicals to induce the SOS response, most of which are potental mutagens and carcinogens, to induce the *umu* operon. A plasmid (pKS1002) carrying a fused gene (*umuC′-′lacZ*) was introduced into *S. typhimurium* TA1535. The Ames test is based on the frequency of reverse mutation (His^+ colonies as spontaneous His^+ revertant colonies). The strain TA100 has a base-pair change mutation. Therefore, it was suggested that **1** is an antimutagenic compound, and **1**-Ac is a desmutagenic compound.

Previously, many antimutagenic compounds were already isolated from plant extracts. For example, Matuo et al. (1994) reported on isolation and identification of (+)-gallocatechin as a bio-antimutagenic compound from leaves of *Psidium guava* Linn. Nakamura et al. (1993) reported on the antimutagenic activity of *S*-methylmethanethiosulfonate from cauliflower (*Bassica oleracea* L. var. *botrytis*). Wall et al. (1988) reported on antimutagenic homoisoflavones (intricatin and intricatinol) from the roots of *Hoffmanosseggia intricata* Brandegee. Okuda et al. (1984) reported on inhibitory effects of tannins included in *Geranium thunbergii*. Many antimutagenic compounds have various skeletons, and these suppressive compounds were examined to elucidate the mechanism of suppressive effects in various assay systems.

In the investigation of the other constituents of *P. praeruptorum*, several coumarin glycosides have been isolated, for example, isorutarin, rutarin, marmesinin, and skimmin (Okuyama et al., 1989). Previously, compound **1** was isolated from several other plants, for example, *Cnidium officinale* (Umbelliferae), *Schefflera digitata* (Araliaceae), and *Heteromorpha trifoliata* (Umbelliferae) (Kobayashi et al., 1987; Muir et al., 1982; Villegas et al., 1988). Compound **1** has been identified as a potent preformed inhibitor and a phytoalexin for many fungal parasites, and it was also found to be neurotoxic for mice and a plant resistance factor against nematodes (Städler et al., 1984). Compound **1**-Ac is also known to have antifungal activity (Muir et al., 1982). There have also been reports on falcarinol, another polyacetylenic compound, the structure of which is closely related to falcarindiol, as an anticancer agent and having antitumor activity (Fujihashi et al., 1992; Matsumoto et al., 1991). However, no reports on the antimutagenicity of falcarindiol have appeared.

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