JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification of Geranic Acid, a Tyrosinase Inhibitor in Lemongrass (*Cymbopogon citratus*)

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Lemongrass is a popular Asian herb having a lemon-like flavor. Very recently, potent tyrosinase inhibitory activity has been found in lemongrass in addition to various biological activities reported in the literature. The aim of the present study is to identify the active compounds in the lemongrass. An assay-guided purification revealed that one of the active substances was geranic acid. Geranic acid has two stereoisomers, which are responsible for the trans and cis geometry on the conjugated double bond. Both isomers are present in the active ethyl acetate-soluble extract of the lemongrass, and their IC_{50} values were calculated to be 0.14 and 2.3 mM, respectively. The structure requirement of geranic acid for the potent tyrosinase inhibitory activity was investigated using geranic acid-related compounds.

KEYWORDS: Lemongrass; geranic acid; *Cymbopogon citratus*; tyrosinase inhibition; enzymatic oxidation

INTRODUCTION

The oxidative deterioration of food involves two components, which are nonenzymatic and enzymatic oxidations. Nonenzymatic oxidation is observed in all foods. It is induced by light irradiation, the redox reaction of a transition metal, and degradation of the oxidized impurity (*1*). This oxidation can be protected by antioxidants. Enzymatic oxidation is observed in fresh foods such as vegetables, fruits, crops, and marine products. The oxidation proceeds by catalytic action of a food-bearing oxidation enzyme and makes rapid progress during food processing (*2*). This oxidation can be prevented by using a typical inhibitor for the respective enzyme. The control of both types of oxidations is very important to preserve the food quality. Tyrosinase is one of the oxidation enzymes widely distributed in fresh foods. It catalyzes the oxidation of the phenolic constituents that results in the browning of food, which reduces the market value of food. To control the harmful tyrosinase activity, the development of an efficient inihibitory substance is required. Many tyrosinase inhibitors have already been developed by synthesis or isolation from natural sources (*3, 4*), because the tyrosinase inhibitor is applicable not only for food but also for cosmetics such as a skin-whitening agent. Recently, consumers have shown a preference for natural substances as safe food ingredients; therefore, various useful agents for food should be sought in natural resources. We have screened the tyrosinase inhibitory activity of 53 parts of 36 plant species (*5*), which were cultivated for traditionally edible and medicinal uses in the subtropical Okinawa area of Japan (*6*). From the screening, lemongrass was selected as one of the potent active species.

Lemongrass (*Cymbopogon citratus*) is widely used as an essential ingredient in tropical Asian cuisine due to its lemon-like flavor (*7*). In Japan, tea prepared from the dry leaves of lemongrass is taken for physical and mental health promotion. Although various bioactivities of lemongrass and their application studies have already been reported [antibacterial (*8–15*), antifungal (*16–18*), mosquito repellent (*19*), insecticide (*20*), cytotoxic (*21*), anti-inflammatory (*22*), and antioxidant (*23*) activities], its potent tyrosinase inhibitory activity has been newly observed in our studies (*5*). We report in this paper the identification of the tyrosinase inhibitor in lemongrass and structural requirement of the inhibitor for activity presentation.

MATERIALS AND METHODS

Materials and Instruments. Dry leaves of lemongrass (*C. citratus*) were purchased from Mikuni Co. Ltd. (Osaka, Japan). Tyrosinase (from mushroom), L-DOPA (L-3,4-dihydroxyphenylalanine), geranic acid (purity = 85%), and activated manganese dioxide were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Kojic acid, dicyclohexylcarbodiimide, and silver oxide were obtained from Wako Pure Chemicals (Osaka, Japan). Farnesol, 3-methylcrotonic acid, crotonic acid, tiglic acid, angelic acid, and methacrylic acid were purchased from Tokyo Kasei (Tokyo, Japan). Silica gel for column chromatography (silica gel 60 no. 9385) and silica gel TLC plates (Kieselgel 60 F254) were purchased from Merck, Japan (Tokyo, Japan). All other reagents and solvents were purchased from Nacalai Tesque (Kyoto, Japan). EI-MS spectra were measured with an SX-102A spectrometer (JEOL, Tokyo, Japan) or a GCQ gas chromatograph–mass spectrometer (Therm Fisher Scientific, Yokohama, Japan). NMR spectra were recorded on an EX-400 spectrometer (JEOL).

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10.1021/jf072893l CCC: $40.75
Published on Web 12/15/2007

Tyrosinase Inhibition Assay. The tyrosinase inhibitory activity was evaluated by using L-DOPA. The 96-well microplate method previously reported by Masuda (*24*) was employed. Briefly, eight wells in total were designated A (three wells), B (one well), C (three wells), and D (one well), which contained the following reaction mixtures: A, 120 μL of a 1/15 M phosphate buffer (pH 6.8) and 40 μL of tyrosinase (46 units/mL) in the same buffer; B, 160 μL of the same buffer; C, 80 μL of the same buffer, 40 μL of tyrosinase (46 units/mL) in the same buffer, 40 μL of an appropriate amount of the sample−buffer solution containing 5% DMSO to dissolve the sample; D, 120 μL of the same buffer and 40 μL of the same amount of the sample solution containing 5% DMSO. As a positive control experiment, kojic acid (final concentration = 0.03 mM) was used for a reference sample. The contents of each well were mixed and then incubated at 23 °C for 10 min, before 2.5 mM L-DOPA in the same buffer was added. After incubation at 23 °C for 2 min, the absorbance at 475 nm of each well was measured. The percentage inhibition of the tyrosinase activity was calculated according to the following equation: $\{[(A - B) - (C - D)]/(A - B)\} \times 100$. In the experiment for crude samples such as extract and fraction, the addition orders of tyrosinase and L-DOPA was inverted, because some phenolics in the extract reacted with tyrosinase to interfere the oxidation of L-DOPA.

Extraction and Fractionation. The dry leaves of lemongrass (4 kg) were soaked in methanol (36 L) at room temperature for 2 weeks. After filtration, the filtrate was evaporated under reduced pressure to give a methanol extract (434 g). The extract (5.1 g) was partitioned with hexane (50 mL) and methanol/water (9:1, 50 mL) to give a hexane-soluble fraction and a methanol-soluble fraction. After removal of the solvent, the methanol-soluble fraction was suspended in water (50 mL) and sequentially extracted with ethyl acetate (50 mL) and *n*-butanol (50 mL) to give an ethyl acetate-soluble fraction and a butanol-soluble fraction, respectively. After evaporation, the yields of these fractions were 1.5 g for the hexane extract, 1.3 g for the ethyl acetate extract, and 0.7 g for the butanol extrtact. The remaining water layer was evaporated to give the water extract (1.6 g). These extracts were used for inhibitory assay.

From 200 g of the methanol extract was prepared 54 g of an active ethyl acetate extract according to the same procedure as described above. Ten grams of the ethyl acetate extract was loaded on a silica gel column (600 mL) and eluted stepwise with 2.5% methanol in chloroform (1.8 L), 5% methanol in chloroform (1.8 L), 10% methanol in chloroform (1.8 L), 20% methanol in chloroform (1.2 L), and 30% methanol in chloroform (1.2 L). The eluate was collected in every ca. 70 mL, and the obtained fractions (total of 109 fractions) were combined to 21 fractions according to the similarity analysis for the constituents of each fraction using silica gel TLC. The 21 fractions were subjected to inhibitory assay after evaporation.

Isolation of Tyrosinase Inhibitory Substance. The most active fraction 5 (1.1 g) of the above-mentioned silica gel chromatography was purified again by silica gel column chromatography eluted with ethyl acetate/hexane (1:4) to give 0.27 g of a fraction containing active substance. To remove the lipidic impurity, 123 mg of the fraction was purified by Sephadex LH-20 column chromatography eluted with isopropanol. Finally, silica gel TLC purification (ethyl acetate/hexane = 1:5) gave active compounds **1** (14 mg, $R_f = 0.36$) and **2** (5.5 mg, $R_f = 0.54$).

1 (*trans*-geranic acid): EI-MS, *m/z* 168 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 1.59 (3H, s, H-8 or 9), 1.66 (3H, s, H-8 or 9), 2.12 (3H, s, H-10), 2.13 (4H, m, H-4 and 5), 5.04 (1H, br s, H-6), 5.66 (1H, br s, H-2), 12.0 (1H, br, COOH); ^{13}C NMR ($CDCl_3$) δ 17.7 (C-9 or 10), 19.1 (C-9 or 10), 25.7 (C-8), 26.0 (C-5), 41.2 (C-4), 115.1 (C-2), 122.8 (C-6), 132.7 (C-7), 163.1 (C-3), 172.1 (C-1).

2 (*cis*-geranic acid): EI-MS *m/z* 168 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 1.58 (3H, s, H-8 or 9), 1.66 (3H, s, H-8 or 9), 1.91 (3H, s, H-10), 2.14 (2H, dt, J = 8.0 and 7.0 Hz, H-5), 2.62 (2H, t, J = 8.0 Hz, H-4), 5.12 (1H, br t, J = 7.0 Hz, H-6), 5.66 (1H, br s, H-2).

Preparation of *trans*- and *cis*-Geranic Acids (1 and 2). Commercially available crude geranic acid (a mixture of mainly trans and cis isomers) was separated by silica gel TLC (5 mg of sample loaded on a 20 cm × 20 cm plate), which was developed twice with ethyl acetate/hexane (1:5). From 44 mg of crude geranic acid were obtained pure *trans*-geranic acid (**1**, 21 mg) and *cis*-geranic acid (**2**, 20 mg).

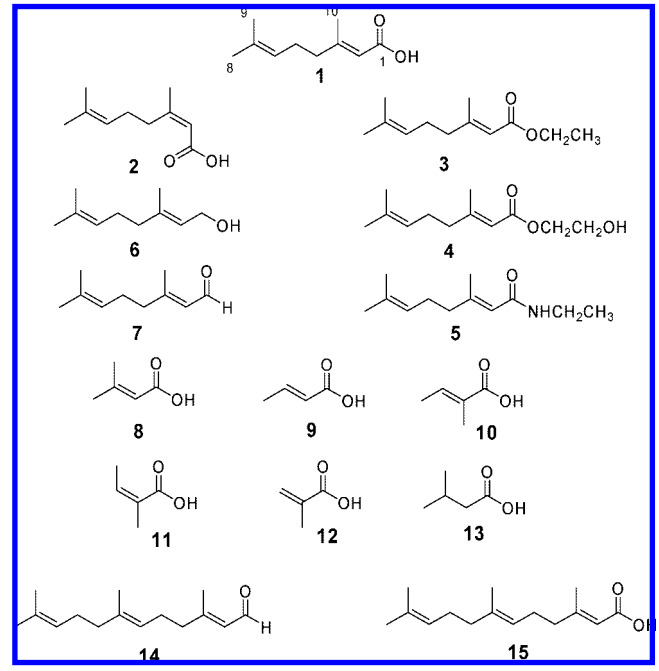

Figure 1. Chemical structures of geranic acid and related compounds.

Preparation of Ester Derivatives of Geranic Acid. To a solution of geranic acid (201 mg, a mixture of trans and cis isomers) in dichloromethane (7 mL) were added dimethylaminopyridine (73 mg), dimethylaminopyridine hydrochloride (95 mg), ethanol (0.6 mL), and dicyclohexylcarbodiimide (495 mg), sequentially. After 1 h of stirring at 23 °C, acetic acid (0.2 mL) and methanol (0.4 mL) were added to the mixture. After the addition of diethyl ether (40 mL), the produced precipitate was filtered off. The filtrate was concentrated and subjected to silica gel column chromatography eluted with chloroform/hexane (1:6) to give geranic acid ethyl ester (**3**, 194 mg, 2:1 mixture of trans and cis isomers): EI-MS, *m/z* 196 $[M]^+$; ^{1}H NMR (trans isomer) ($CDCl_3$) δ 1.28 (3H, t, J = 7.5 Hz), 1.59 (3H, s), 1.67 (3H, s), 2.01 (7H, br s), 4.14 (2H, q, J = 7.5 Hz), 5.06 (1H, m), 5.65 (1H, s).

A similar procedure using ethylene glycol instead of ethanol was employed to synthesize geranic acid ethylene glycol ester (**4**, 76 mg, 2:1 mixture of trans and cis isomers) from geranic acid (105 mg): EI-MS, *m/z* 212 $[M]^+$; ^{1}H NMR (trans isomer) ($CDCl_3$) δ 1.60 (3H, s), 1.68 (3H, s), 2.15 (7H, br s), 3.84 (2H, m), 4.21 (2H, m), 5.07 (1H, m), 5.71 (1H, s).

Preparation of an Amide Derivative of Geranic Acid. To the hydroxysuccinimide ester of geranic acid (100 mg), which was prepared from geranic acid, hydroxysuccinimide, and dicyclohexylcarbodiimide, was added a chloroform solution (6 mL) of ethylamine hydrochrolide (154 mg) and triethylamine (0.2 mL). After 20 min of stirring at 23 °C, the mixture was evaporated and subjected to silica gel column chromatography eluted with ethyl acetate/hexane (1:4) to give geranic acid ethyl amide (**5**, 32 mg, 2:1 mixture of trans and cis isomers): EI-MS, *m/z* 195 $[M]^+$; ^{1}H NMR (trans isomer) ($CDCl_3$) δ 1.14 (3H, t, J = 7.2 Hz), 1.59 (3H, s), 1.68 (3H, s), 2.03–2.18 (4H, m), 2.12 (3H, s), 3.30 (2H, q, J = 7.2 Hz), 5.06 (1H, m), 5.33 (1H, br), 5.53 (1H, s).

Preparation of Geranial and Farnesyl Aldehyde. To a solution of geraniol (**6**, 306 mg) in hexane (15 mL) was added activated manganese dioxide (4 g). After 4 h of stirring at room temperature, the manganese dioxide was filtered off. The obtained filtrate was evaporated and subjected to silica gel column chromatography eluted with chloroform/hexane (1:1) to give geranial (**7**, 222 mg): EI-MS, *m/z* 152 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 1.62 (3H, s), 1.68 (3H, s), 2.17–2.28 (4H, m), 2.17 (3H, s), 5.07 (1H, m), 5.88 (1H, br t, J = 7.5 Hz), 9.99 (1H, d, J = 7.5 Hz).

According to a similar procedure, farnesyl aldehyde (**14**, 281 mg) was synthesized from farnesol (400 mg): EI-MS, *m/z* 220 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 1.59 (6H, s), 1.67 (3H, s), 1.98 (2H, m), 2.04 (2H, m), 2.15–2.28 (4H, m), 2.17 (3H, s), 5.08 (2H, m), 5.84 (1H, br d, J = 8.5 Hz), 9.98 (1H, d, J = 8.5 Hz).

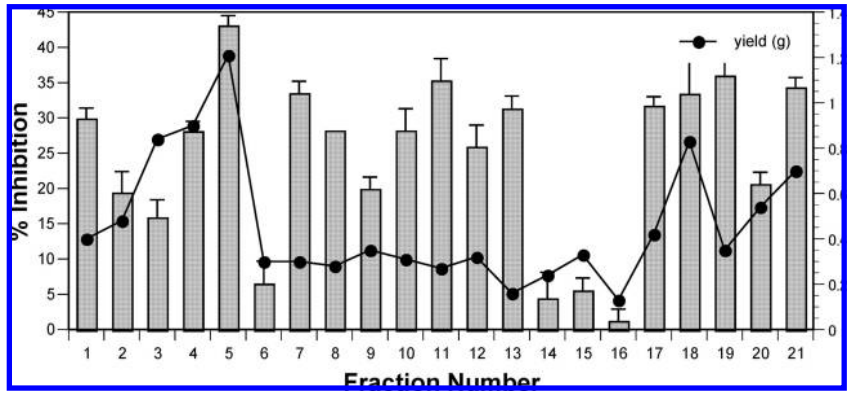

Figure 2. Tyrosinase inhibitory activity and yield of the fractions by silica gel column chromatography of the ethyl acetate extract of the lemongrass. The examined concentration of each fraction was 0.05 mg/mL, and the data were obtained in triplicate and averaged. Each SD value is expressed by an error bar.

Table 1. Tyrosinase Inhibitory Activity (Percent Inhibition) of Geranic Acid and Related Compounds[a]

compound	concentration					IC_{50} (mM)
	0.03 mM	0.1 mM	0.3 mM	1 mM	3 mM	
trans-geranic acid (**1**)	21.8 ± 1.8	43.6 ± 0.0	64.2 ± 1.0	82.4 ± 1.0	−	0.14
geranic acid ethyl ester (**3**)	−	−	1.8 ± 3.5	4.7 ± 2.7	−	#
geranic acid ethylene glycol ester (**4**)	−	−	−8.3 ± 2.9	7.1 ± 2.2	−	#
geranic acid ethyl amide (**5**)	−	−	−	−3.3 ± 2.3	0.7 ± 3.0	#
geraniol (**6**)	−	−	−	−0.7 ± 1.2	−1.4 ± 3.2	#
geranial (**7**)	−	8.0 ± 1.0	17.8 ± 2.6	41.4 ± 4.6	60.3 ± 0.0	1.62
3-methylcrotonic acid (**8**)	−	−	14.7 ± 1.1	39.7 ± 5.6	61.5 ± 0.0	1.70
crotonic acid (**9**)	−	−	−	4.5 ± 7.8	8.3 ± 2.9	#
tiglic acid (**10**)	−	−	−	−3.0 ± 3.4	18.5 ± 1.3	#
angelic acid (**11**)	−	−	−	−9.6 ± 1.9	−9.0 ± 1.1	#
methacrylic acid (**12**)	−	−	−	−7.7 ± 0.0	−1.3 ± 2.2	#
isovaleric acid (**13**)	−	−	−	4.3 ± 2.8	7.4 ± 1.9	#
farnesic acid (**15**)	23.0 ± 1.0	47.9 ± 1.0	70.9 ± 0.0	100 ± 0.0	−	0.11

[a] −, not determined; #, could not be calculated.

Preparation of Farnesic Acid (All-trans Isomer). To a solution of farnesyl aldehyde (**14**, 305 mg) in methanol (10 mL) were added sodium cyanate (340 mg), acetic acid (119 μL), and activated manganese dioxide (2.4 g). After 3 h of stirring at 23 °C, manganese dioxide was filtered off. The filtrate was poured into ethyl acetate and saturated NaCl aqueous solution and extracted three times with ethyl acetate. The combined ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated. The residue was subjected to silica gel chromatography eluted with chloroform/hexane (1:1) to give the methyl ester of farnesic acid (a mixture of all-trans and 2-cis isomers, 184 mg). To the ester (105 mg) in methanol (5.5 mL) was added 1 N NaOH aqueous solution. After 3 h of stirring at 60 °C, the mixture was poured into 1 N HCl aqueous solution, extracted three times with chloroform, and evaporated. The residue was purified by silica gel column chromatography eluted with ethyl acetae/hexane (1:6) to give a cis and trans mixture of farnesic acid (54 mg). Twenty milligrams of the mixture was purified by silica gel TLC (ethyl acetate–/hexane = 1:10) to give *all-trans*-farnesic acid (**15**, 14 mg): EI-MS, *m/z* 236 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 1.60 (6H, s), 1.70 (3H, s), 1.98 (2H, m), 2.05 (2H, m), 2.20 (4H, br), 2.20 (3H, s), 5.09 (2H, br), 5.70 (1H, s).

RESULTS AND DISCUSSION

Fractionation of the Extract of Lemongrass and Isolation of Tyrosinase Inhibitors 1 and 2. The dry leaves of the lemongrass (4 kg) were extracted with methanol, because the alcoholic extract of the lemongrass showed a potent tyrosinase inhibitory activity (*5*). The methanol extract (5.1 g) was next separated into four extracts by a solvent partition method, and the tyrosinase inhibitory activity of the extracts was measured. Three organic solvent-soluble extracts (H, E, and B) showed an inhibitory activity at the concentration of 0.15 mg/mL (inhibitory percent for H, 28.7 ± 7.2; E, 62.5 ± 0; B, 55.2 ± 3.5), whereas the water-soluble extract (W) promoted activity (inhibitory percent for W, −41.4 ± 0). The ethyl acetate (E)- and butanol (B)-soluble extracts showed potent activities comparable to that of 0.03 mM kojic acid (inhibitory percent = 70.1 ± 2.0). Thus, the most active ethyl acetate-soluble extract was selected for the purification of the tyrosinase inhibitor of the lemongrass. The ethyl acetate extract (54 g) was prepared again from 200 g of the methanol extract according to the same procedure, and 10 g of the extract was subjected to silica gel column chromatography to separate into 21 fractions. The yield and inhibitory activity of each fraction are summarized in **Figure 1**. **Figure 1** shows that many fractions have a moderate inhibitory activity at the concentration of 0.05 mg/mL. Among the active fractions, the activity of fraction 5 was the strongest and the yield was also the highest. The contribution of each fraction to the activity of the plant should be estimated using the value obtained by multiplication of each yield and activity; therefore, fraction 5 should be selected as the fraction having the greatest contribution and subjected to further investigation. The constituents of fraction 5 were purified by subsequent silica gel column chromatography, Sephadex LH-20 column chromatography, and silica gel TLC, to isolate the major compound **1** and the minor compound **2** as pure forms.

Structure Identification and Tyrosinase Inhibitory Activities of 1 and 2. Compound **1** was isolated as a colorless oil. Its molecular weight, which was obtained by EI-MS measurement, and typical ^{1}H and ^{13}C NMR data, revealed that **1** was identical to *trans*-geranic acid (*25*). Compound **2** was also isolated as a

colorless oil and showed the same molecular ion value as that of **1**. Analysis of ^{1}H NMR data of **2** with comparison of that of **1** revealed that **2** was a cis isomer at the 2-olefin of **1** (*25*). Therefore, **1** and **2** were identified as *trans*- and *cis*-geranic acids, respectively (**Figure 2**).

The tyrosinase inhibitory activity of the *trans*- and *cis*-geranic acids (**1** and **2**) was measured. *trans*-Geranic acid showed a clear dose–response curve between 0.03 and 1 mM, and its IC_{50} value was estimated to be 0.14 mM. Although the *cis*-geranic acid also showed a dose–response curve, the activity of **2** was much weaker than that of **1** (IC_{50} = 2.3 mM). The activities of these geranic acids were weaker than that of kojic acid (IC_{50} = 0.017 mM), a potent tyrosinase inhibitor; however, the tyrosinase inhibitory property of geranic acid was first reported in this paper.

Tyrosinase Inhibitory Activity of Geranic Acid Related Compounds. Although geranic acid is a simple compound, the stereochemical difference observed in the isolated trans and cis isomers affects the inhibitory activity of geranic acid. A question arose as to which part of the geranic acid structure was important for the appearance of the tyrosinase inhibitory activity. Therefore, the activity-required structure of geranic acid was analyzed using several synthetic and commercially available analogs (**Figure 2**). The percent inhibiton values of 12 geranic acid-related compounds are summarized in **Table 1**. Geranial (**7**), which was an aldehyde analogue of *trans*-geranic acid (**1**), showed a lower activity than *trans*-geranic acid. Geraniol (**6**), an alcohol analogue of *trans*-geranic acid, did not show any activity under the examined conditions. The ester and amide derivatives (**3**, **4**, **5**), which were synthesized from geranic acid, showed no activity. These results indicated that the carboxylic acid moiety of geranic acid was very important for its activity. The tyrosinase inhibitory activity of various aromatic carboxylic acids has been reported (*26–30*); however, these activity strengths were lower than those of the structurally related aldehyde derivatives (*27, 28*). In the case of the geranic acid structure, the carboxylic acid [*trans*-geranic acid (**1**), IC_{50} = 0.14 mM] was stronger than that obtained for the aldehyde [geranial (**7**), IC_{50} = 1.62 mM] (*31*). Most aldehyde inhibitors are believed to show their activity by forming a stable Schiff base with a primary amino group of the enzyme (*32*). On the other hand, the carboxylic acids are suggested to inhibit by their coordination to the copper of the binuclear site of the enzyme (*33*). Geranic acid may have a structural part, which enhances the coordination to the active site of the enzyme.

Next, to investigate the effect of the alkyl part adjacent to the conjugated double bond (3-position) of geranic acid, farnesic acid (**15**) was synthesized. Elongation of the C6 unit at the 3-position of geranic acid to the C11 unit, which appeared on the fernesic acid, slightly increased the inhibitory activity (IC_{50} = 0.11 mM of **15** from 0.14 mM of **1**). The reduction effect of the C6 unit to the methyl group, which was examined using 3-methylcrotonic acid (**8**), reduced the activity (IC_{50} = 1.70 mM). These results indicated that a longer alkyl chain adjacent to the 3-position was effective, but not drastic. The appropriate hydrophobic part of the enzyme inhibitor is well-known to strengthen the activity by stabilizing its interaction with the enzyme. For the tyrosinase inhibition, a similar effect has also been observed (*31, 34*). In the case of geranic acid, the alkyl part at the 3-position may contribute to this stabilization of the enzyme–inhibitor interaction. Although the activity of 3-methylcrotonic acid (**8**) was weaker than that of *trans*-geranic acid, it still has an attractive activity (IC_{50} = 1.70 mM). Therefore, the structural effect around the conjugated double bond at the 2-position was examined using the 3-methylcrotonic acid analogues. The conjugated double bond at the 2-position was found to be essential because isovaleric acid (**13**), the single-bond derivative of **8**, showed almost no activity. Reduction or rearrangement of the methyl substitution on the double bond of **8** always reduced the activity, which was revealed from the weak activities of crotonic acid (**9**), tiglic acid (**10**), angelic acid (**11**), and methacrylic acid (**12**). This double-bond moiety would cause a steric interaction with the tertiary structure of the active site of tyrosinase when the carboxylic acid part is coordinated to the active site. Therefore, the observed structural restriction around the double bond would be required to increase the inhibitory activity of geranic acid.

Geranic acid is a structurally simple tyrosinase inhibitor from lemongrass; however, the very restricted structure of a long-alkyl-substituted trans-conjugated carboxylic acid was estimated as an essential structure for the potent tyrosinase inihibitory activity.

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Received for review October 1, 2007. Revised manuscript received November 13, 2007. Accepted November 18, 2007. This work was financially supported by Kieikai Research Foundation (Tokyo, Japan).

JF072893L