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Flavokawains B and C, melanogenesis inhibitors, isolated from the root of *Piper methysticum* and synthesis of analogs

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ABSTRACT

The ethanolic extract of the root of *Piper methysticum* was found to inhibit melanogenesis in MSH-activated B16 melanoma cells. Flavokawains B and C were isolated from this extract based on their anti-melanogenesis activity and found to inhibit melanogenesis with IC_{50} values of 7.7 μM and 6.9 μM, respectively. Flavokawain analogs were synthesized through a Claisen–Schmidt condensation of their corresponding acetophenones and benzaldehydes and were evaluated in terms of their tyrosinase inhibitory and anti-melanogenesis activities. Compound **1b** was the most potent of these with an IC_{50} value of 2.3 μM in melanogenesis inhibition assays using MSH-activated B16 melanoma cells.

Melanin is a natural substance, synthesized by specialized cells known as melanocytes, that gives color to hair, skin, eyes, and other body features. The major function of melanin is to protect against the damaging effects of ultraviolet radiation. However, excessive synthesis and accumulation of melanin in the skin can lead to a number of dermatologic disorders, including melasma, solar lentigo, and age spot.[1] Melanin biosynthesis can be reduced by avoiding UV exposure, inhibiting melanocyte metabolism, and regulating melanogenic enzymes or the uptake and distribution of melanosomes in recipient melanocyte.[2] While many melanogenesis inhibitors have been reported and are used as cosmetic additives, many other known compounds suffer from high toxicity, low stability, poor skin penetration, and/or insufficient activity. For example, consumer application of arbutin, kojic acid, and various synthetic agents are limited by serious side effects in humans.[3] Therefore, recent research on melanogenesis inhibitors has focused on the use of natural products in the cosmetics industry.[4]

Piper methysticum, or kava, of the family Piperaceae, grows as a perennial shrub in the South Pacific islands.[5] The root of kava is commonly used by native Pacific islanders to prepare a beverage for ceremonial activities. Commercially, kava has been used in natural remedies for insomnia, anxiety, and menopausal symptoms. However, the consumption of kava has recently been reported to cause serious side effects such as hepatitis and acute liver failure.[6] As a result, products containing kava extracts were banned in a number of European countries, the USA, and Canada.[7]

During our investigation of melanogenesis inhibitors derived from natural sources for use as constituents of cosmetic products and depigmenting agents, we discovered that a crude ethanolic extract of kava root exhibited as moderate inhibitory activity on melanogenesis in MSH-stimulated B16 melanoma cells.

This Letter describes the inhibitory effects of flavokawain B (**1a**) and flavokawain C (**1d**), isolated from *Piper methysticum* on melanogenesis. We also investigated the structural requirements for this inhibitory activity through the synthesis of new flavokawain analogs and evaluation of their inhibitory activity on melanogenesis.

Kava root (100 g), collected in Micronesia, was dried, ground, and extracted with aqueous EtOH (80%) for 48 h. After filtering, the brown extract solution was evaporated in vacuo to afford a brown residue (8.5 g). The residue was then partitioned between methanol and hexane. The hexane layer was evaporated in vacuo to give a 'hexane fraction' (1.5 g). The MeOH-soluble layer was dried and diluted with distilled water, and further partitioned by

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successive solvent extractions with ethyl acetate and n-butanol. Each fractions was evaporated in vacuo to yield an 'ethyl acetate fraction' (7.7 g), an '*n*-butanol fraction' (0.15 g), and an 'H_2O fraction' (0.4 g). The 'hexane fraction' was most effective in inhibiting melanogenesis in B16 melanoma cells (52% inhibition at 10 μg/ml). This fraction was separated through a silica column to obtain six fractions, hexane-1 to -6. Each of these was tested in terms of the inhibition of melanogenesis. Fractions hexane-4 and hexane-6 exhibited inhibitory effects on melanin production in B16 melanoma cells (Table 1).

To identify compounds responsible for the observed inhibitory activities, two major compounds were isolated from hexane-4, and one compound from hexane-6, by silica column. These compounds isolated from hexane-4 were identified as flavokawain A and flavokawain B, and the compound isolated from hexane-6 was identified as flavokawain C by NMR and mass spectroscopic analyze. Their physicochemical spectroscopic data of the isolated compounds matched published values.[8]

Flavokawains are a class of chalcone found in kava plant. Currently flavokawain A, B, and C were identified (Fig. 1).

For confirming the inhibitory effects of flavokawain A, B, and C on melanogenesis, the production of melanin in MSH-activated B16 melanoma cells was tested with a kojic acid as positive control. Flavokawain B and C inhibited the production of melanin in a dose-dependent manner with IC_{50} values of 7.7 and 6.9 μM, respectively. However, flavokawain A exhibited no inhibitory activity despite its structural similarities with flavokawain B and C. These result indicated that the inhibitory activity of hexane-4 must result from the presence of flavokawain B, and that of hexane-6 from the presence of flavokawain C. Cell viability assays indicated that the observed reduction in melanine production was not caused by cytotoxic effects of these compounds.[9]

Recently, several chalcones have been reported as potential inhibitors of tyrosinase. The position of the hydroxyl group on the chalcone rings was shown to be important for that activity. In 2007, Jun reported 2′,4′,6′-trihydroxy chalcones as a new class of tyrosinase inhibitors. In particular, trihydroxy chalcones, which have a 2,4-hydroxy resorcinol structure on their B-ring, were potent inhibitors of mushroom tyrosinase with an IC_{50} = 1 μM.[10] Based on these Letters, we hypothesized that the inhibitory activities of flavokawain B and C on melanogenesis result from tyrosinase inhibition. However, the flavokawains did not inhibit mushroom tyrosinase.[11] This indicates that both flavokawain B and C inhibit melanogenesis without inhibiting tyrosinase.

We hypothesized that the hydroxyl or methoxyl substituent on the B ring of a chalcone derivative would be a key pharmacophore for anti-melanogenesis.

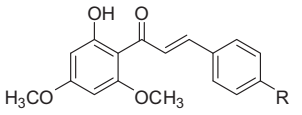
To investigate the structure–activity relationship (SAR) of chalcone B-ring, derivatives **1a**–**1n** were synthesized through a Claisen–Schmidt condensation of the corresponding acetophenones and benzaldehydes with yields of 60–90%.

The general procedure for these reactions was as follows.

Table 1
Inhibitory effects of hexane-1–6 from *Piper methysticum* on melanogenesis in B16 melanoma cells

Samples	Inhibition rate of melanogenesis[a] (%)	
	3 μg/ml	10 μg/ml
Hexane-1	21 ± 15	23 ± 12
Hexane-2	27 ± 17	26 ± 12
Hexane-3	40 ± 24	27 ± 17
Hexane-4	46 ± 22	>99 ± 10
Hexane-5	26 ± 4	24 ± 10
Hexane-6	45 ± 15	>99 ± 11

[a] Values are the of at least two measurements.



R = OCH_3 flavokawain A (**1i**)
H flavokawain B (**1a**)
OH flavokawain C (**1d**)

Figure 1. Flavokawains isolated from *Piper methysticum*.

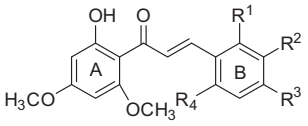
A solution of acetophenone (1.2 mmol) and aldehyde (1.4 mmol) in methanol (3 ml) was added dropwise under nitrogen to a stirred solution of KOH (26.7 mmol) in water (1.5 ml) that had been cooled to 0 °C in an ice bath. The reaction mixture was held at 0 °C for 3 h and then at room temperature for 2 days. The mixture was poured into ice-water, adjusted to pH 3–4 with 1 M HCl, and then extracted with ethyl acetate. The organic layer was successively washed with water and saturated brine, and dried over anhydrous Na_2SO_4. After concentration under reduced pressure, the resultant solid was recrystallized from a solution hexane and ethyl acetate.

Chalcones (**1a**–**1n**) were first evaluated for anti-melanogenic activities in B16 melanoma cells at 10 ppm. Compounds exhibiting with >50% inhibition 10 ppm were further evaluated other concentrations to determine IC_{50} values (Table 2). Cell viability assays of all synthesized chalcones in Table 2 indicated that the observed reduction in melanine production was not caused by cytotoxic effects of these compounds. As shown in Table 2, most of the chalcones were potent inhibitors of melanogenesis.

Compounds **1b**–**1d** have monohydroxyl substituent at *o*-, *m*- or *p*-position of the B-ring. *o*-Hydroxy substituted compound **1b** was the most potent inhibitor, with an IC_{50} value of 2.3 μM. The *meta*- and *para*-substituted derivatives had IC_{50} value of 9.9 and 6.9 μM, respectively. Among the monomethoxy substituents, the *ortho*- and *meta*-substituted compounds **1g** and **1h** showed inhibitory activities with IC_{50} values of 3.8 and 3.1 μM, respectively. However, the *para*-methoxy-substituted compound **1i** showed no inhibitory activity. Flavokawain B (**1a**), which does not have a substituent on its B-ring, showed a moderate inhibitory activity with an IC_{50} of 7.7 μM. To further investigate the effect of hydroxyl substituents, dihydroxylated compounds **1e** and **1f** were evaluated in terms of their melanogenesis inhibitory activity. The introduction of a hydroxyl group at the *o*-position on the B-ring of flavokawain C (**1d**) played an important role in its enhancing the activity (**1d** → **1f**, IC_{50} 6.9 μM → 3.4 μM). However, an additional *m*-hydroxy group decreased this activity (**1d** → **1e**, IC_{50} 6.9 μM → 31.6 μM). SAR studies revealed that the introduction of *p*-methoxy functional groups on the B-ring (**1i** and **1j**) leads to no activity. In contrast, the introduction of *o*-hydroxy or methoxy group on the B-ring has a considerable effect on their potency. To evaluate the effect of *o*-substituent on B-ring, novel compounds with *o*-alkyl group were synthesized and tested.[12] Compared to flavokawain B (**1a**), the introduction of alkyl group generally increased activity (**1a** → **1l**, **1m**, **1n**: IC_{50} 7.7 μM → 3.0, 2.5, 2.7 μM). However, in the case of an *o*-dimethyl compound (**1k**), the additional *o*-methyl group led to a decrease in activity (**1a** → **1k**: IC_{50} 7.7 μM → 9.9 μM), likely due to steric hindrance by the *o*-dimethyl group.

In conclusion, the ethanolic extract of the root of *Piper methysticum* was found to inhibit melanogenesis in MSH-activated B16 melanoma cells. Flavokawains B and C were isolated from this extract based on their anti-melanogenesis activity and found to inhibit melanogenesis with IC_{50} values of 7.7 and 6.9 μM, respectively. Flavokawain analogs were synthesized through a

Table 2
Tyrosinase inhibitory and anti-melanogenic activities of 2′-hydroxy, 4′,6′-dimethoxychalcones with 2-hydroxyl substituents on B-ring of chalcones on melanogenesis in B16 melanoma cells



Compounds	R^1	R^2	R^3	R^4	Tyrosinase inhibition (IC_{50} μM)	Melanogenesis inhibition (IC_{50} μM)
Kojic acid[12]	—	—	—	—	30	>500
Flavokawain B (**1a**)	H	H	H	H	—	7.7
1b	OH	H	H	H	—	2.3
1c[17]	H	OH	H	H	—	9.9
Flavokawain C (**1d**)	H	H	OH	H	—	6.9
1e[10]	H	OH	OH	H	—	31.6
1f[17]	OH	H	OH	H	—	3.4
1g[13]	OCH_3	H	H	H	—	3.8
1h[14]	H	OCH_3	H	H	—	3.1
Flavokawain A (**1i**)	H	H	OCH_3	H	—	—
1j[15]	OCH_3	H	OCH_3	H	—	—
1k[17]	CH_3	H	H	CH_3	—	9.9
1l[16]	CH_3	H	H	H	—	3.0
1m[17]	CH_2CH_3	H	H	H	—	2.5
1n[17]	$CH(CH_3)_2$	H	H	H	—	2.7

Claisen–Schmidt condensation of their corresponding acetophenones and benzaldehydes and were evaluated in terms of their tyrosinase inhibitory and anti-melanogenesis activities. All of the isolated flavokawain analogs were inactive in mushroom tyrosinase assays. In particular, flavokawain derivatives were over 20–200 times stronger inhibitors than kojic acid. Compound **1b** was the most potent of these with an IC_{50} value of 2.3 μM in melanogenesis inhibition assays using MSH-activated B16 melanoma cells. Based on structure–activity analysis, we concluded that an *o*-hydroxyl or alkyl group on the B-ring is a pharmacophore for increased inhibitory activity. In contrast, an *m*-methoxyl group on B-ring is a pharmacophore for decreased activity. Further studies on the mechanism of action of flavokawain analogs in melanogenesis are underway.

Acknowledgments

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9. *Measurements of melanin content and cell viability*: Melanin content and cell number were measured in melan-a melanocytes. The cells (2×10^5 cells/ml) were seeded into 24-well plates and the test compounds/fractions were added in triplicate. The medium was charged daily and after 4 d of culture, the cells were lysed with 1 ml of 1 N NaOH. Then 200 μl of each crude cell extract was transferred into 96-well plates. The relative melanin content was measured at 400 nm with a microplate reader (Molecular Devices). Cell viability was determined using a modified crystal violet assay. After removing the medium from each well, the cells were washed with PBS and stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed four times with distilled water, and crystal violet retained by adherent cells was extracted with 95% ethanol at room temperature for 10 min. Crystal violet absorption was measured at 590 nm (Molecular Devices Co., Sunnyvale, CA, USA).
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11. *Measurement of mushroom tyrosinase activity*: Mushroom tyrosinase, L-tyrosine, and L-DOPA were purchased from Sigma Chemical. The reaction mixture for mushroom tyrosinase activity consisted of 150 μl of 0.1 M phosphate buffer (pH 6.5), 3 μl of sample solution, 8 μl of mushroom tyrosinase (2100 unit/ml, 0.05 M phosphate buffer at pH 6.5), and 36 μl of 1.5 mM L-tyrosine or L-DOPA. Tyrosinase activity was determined by reading the optical density at 490 nm on a microplate reader (Bio-Rad 3550, Richnmond, CA, USA) after incubation for 20 min at 37 °C. The inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity (IC_{50}).
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17. *The data of new compounds*: Compound **1c**, yellowish solid, ^{1}H NMR (500 MHz, DMSO-d_6): 13.52 (s, 1H), 9.68 (s, 1H), 7.69 (d, J = 15.4 Hz, 1H), 7.55 (d, J = 15.4 Hz, 1H), 7.24 (t, J = 7.8 Hz, 1H), 7.12 (d, J = 7.5 Hz, 1H), 7.07 (s, 1H), 6.83 (m, 1H), 6.15 (d, J = 1.7 Hz, 1H), 6.12 (d, J = 1.7 Hz, 1H), 3.89 (s, 3H), 3.81 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) 192.3, 165.8, 165.7, 162.0, 157.8, 142.6, 136.1, 130.2, 127.3, 119.8, 117.8, 114.4, 106.3, 93.9, 91.2, 56.3, 55.8; HRMS m/z $[M-H]^-$ 299.0942, calcd for $C_{17}H_{15}O_5$ 299.0919, mp 158–161 °C, compound **1f**, yellowish solid, ^{1}H NMR (500 MHz, DMSO-d_6): 13.88 (s, 1H), 10.23 (s, 1H), 9.99 (s, 1H), 7.90 (d, J = 15.6 Hz, 1H), 7.71 (d, J = 15.6 Hz, 1H), 7.44 (d, J = 8.5 Hz, 1H), 6.37 (d, J = 2.3 Hz, 1H), 6.32 (dd, J = 8.6, 2.3 Hz, 1H), 6.13 (d, J = 2.3 Hz, 1H), 6.09 (d, J = 2.4 Hz, 1H), 3.87 (s, 3H), 3.80 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) 192.6, 165.6, 165.1, 161.8, 161.4, 159.2, 139.7, 130.6, 122.7, 113.6, 108.2, 106.3, 102.6, 93.9, 91.0, 56.1, 55.6; HRMS m/z $[M-H]^-$ 315.0898, calcd for $C_{17}H_{15}O_6$ 315.0868, compound **1k**, yellowish solid, ^{1}H NMR (500 MHz, $CDCl_3$): 7.95 (d, J = 16.0 Hz, 1H), 7.52 (d, J = 16.0 Hz, 1H), 7.11 (m, 3H), 6.11 (d, J = 2.4 Hz), 6.05 (d, J = 2.4 Hz, 1H), 3.85 (s, 3H), 3.81 (s, 3H), 2.42 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) 192.7, 168.5, 166.4, 162.6, 140.8, 137.2, 135.2, 132.9, 106.5, 93.9, 91.3, 55.9, 55.7, 21.4; HRMS m/z $[M+H]^+$ 313.1444, calcd for $C_{19}H_{21}O_4$ 312.1440, mp 81–84 °C, compound **1m**, yellowish solid, ^{1}H NMR (500 MHz, $CDCl_3$): 8.12 (d,

J = 15.4 Hz, 1H), 7.82 (d, *J* = 15.4 Hz, 1H), 7.65–7.67 (m, 1H), 7.32–7.35 (m, 1H), 7.23–7.28 (m, 2H), 6.12 (d, *J* = 2.4 Hz, 1H), 5.97 (d, *J* = 2.4 Hz, 1H), 3.91 (s, 3H), 3.84 (s, 3H), 2.85 (dd, *J* = 7.3, 15.1 Hz, 2H), 1.27 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) 192.7, 168.6, 166.5, 162.8, 144.6, 140.0, 134.1, 130.1, 129.4, 128.8, 126.8, 126.4, 106.5, 93.9, 91.4, 55.9, 55.7, 26.6, 16.0; HRMS *m*/*z* $[M+H]^+$ 313.1446, calcd for $C_{19}H_{21}O_4$ 313.1440, mp 96–99 °C compound **1n**, yellowish solid, ^{1}H NMR (500 MHz, $CDCl_3$): 8.22 (d, *J* = 15.3 Hz, 1H), 7.79 (d, *J* = 15.3 Hz, 1H), 7.62 (dd, *J* = 7.7, 1.2 Hz, 1H), 7.44–7.32 (m, 2H), 7.23 (m, 1H), 6.12 (d, *J* = 2,4 Hz, 1H), 5.96 (d, *J* = 2.4 Hz, 1H), 3.90 (s, 3H), 3.83 (s, 3H), 3.44 (hept, *J* = 6.8 Hz, 1H), 1.29 (d, *J* = 6.9 Hz, 6H) ^{13}C NMR (125 MHz, $CDCl_3$) 192.8, 168.5, 166.4, 162.7, 148.5, 140.1, 133.7, 130.1, 129.3, 126.9, 126.1, 125.6, 106.4, 93.9, 91.4, 55.9, 55.7, 29.3, 23.8; HRMS *m*/*z* $[M+H]^+$ 327.1601, calcd for $C_{20}H_{23}O_4$ 326.1596 mp 92–94 °C.