

## Daedalin A, a Metabolite of *Daedalea dickinsii*, Inhibits Melanin Synthesis in an *in Vitro* Human Skin Model

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The culture broth of *Daedalea dickinsii* was found to predominantly contain the tyrosinase inhibitor, (2*R*)-6-hydroxy-2-hydroxymethyl-2-methyl-2*H*-chromene, daedalin A (**1**). Ongoing research into bioactive metabolites resulted in the identification of two new 2*H*-chromenes, 6-hydroxy-5,7-dimethoxy-2,2-dimethyl-2*H*-chromene (**3**) and 6-hydroxy-2-hydroxymethyl-5-methoxy-2-methyl-2*H*-chromene (**4**), together with 6-hydroxy-2,2-dimethyl-2*H*-chromene (**2**). Comparative studies of isolated compounds **1–4** and related compounds ( $\pm$ )-**1** and **1a–1c** showed **1** to have the strongest tyrosinase inhibitory activity. These results suggest that the hydroxyl groups at positions **6** and **9** of **1** were important for the potent activity. A Lineweaver-Burk plot for a kinetic analysis indicates that **1** competed with L-tyrosine for tyrosinase. Compound **1** also suppressed melanogenesis in a human skin model (up to 49% at 2.8  $\mu$ mol/tissue application) without affecting the cell viability. Compounds **1**, **1b** and **1c** also showed 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity comparable to that of  $\alpha$ -tocopherol.

**Key words:** *Daedalea dickinsii*; chroman; tyrosinase; antioxidant

Tyrosinase is a copper-containing enzyme which catalyzes the oxidation of L-tyrosine and L-3,4-dihydroxyphenylalanine (L-dopa).<sup>1</sup> These oxidation reactions are necessary for melanocytes to synthesize melanin, the compound largely responsible for hair and skin color in mammals. However, an excessive accumulation of melanin in the epidermis causes abnormal hyperpigmentation of the skin such as freckles and lentigines.<sup>2</sup> There has therefore been considerable effort to find an effective tyrosinase inhibitor. Some tyrosinase inhibitors such as arbutin<sup>3</sup> and kojic acid<sup>4</sup> have been used as pharmaceutical constituents in order to prevent hyperpigmentation.

We have recently isolated from a mycelial culture broth of the Polyporaceae fungus, *Daedalea dickinsii*, a

tyrosinase inhibitor, (2*R*)-6-hydroxy-2-hydroxymethyl-2-methyl-2*H*-chromene (**1**) which we named as daedalin A.<sup>5</sup> Several bioactive 2*H*-chromenes have been isolated and shown to have antioxidant, antiviral, antifungal, cytotoxic and anti-inflammatory activities.<sup>6–10</sup> We have reported that the tyrosinase inhibitory activity of **1** was stronger than that of arbutin. Furthermore, **1** also showed potent antioxidant activity. It would therefore be of interest to study the inhibition of melanin synthesis by daedalin A.

An analysis of the mycelial culture broth of *D. dickinsii* by thin-layer chromatography revealed the presence of several compounds in the broth. Our interest in the analogs of **1** isolated from *D. dickinsii* led to the synthesis of several compounds, and we then studied the structure/activity relationship of **1** and the analog compounds. In comparative studies, the most potent tyrosinase inhibitory compound, **1**, also inhibited melanin biosynthesis in human skin.

### Materials and Methods

**General.** (*R*)-trolox, L-tyrosine, L-dopa, tyrosinase and arbutin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A three-dimensional cultured human skin model, Melanoderm (MEL312B), was purchased from Kurabo Co. (Osaka, Japan). The growth medium for the human skin model was a long-life maintenance medium (LLMM), supplied with the MEL312B kit. Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Mycelia of *D. dickinsii* were induced from fruiting bodies collected in Nara, Japan, and have been deposited in the Riken Bioresource Center (deposit number JCM12697, Saitama, Japan).

**Instruments.** Optical rotation values were measured with a JASCO DIP 1000 polarimeter, and mass spectra were measured by a JEOL JMS 700 mass spectrometer. <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were recorded by a Bruker DRX 500 FT-NMR spectrometer, operated at 500.1 MHz for protons and at 125.1 MHz for carbons. TMS was used as the internal standard. IR spectra were taken with a JASCO FT-IR 480 Plus spectrometer.

**Isolation of **2**, **3** and **4**.** *Daedalea dickinsii* mycelia were cultured in a liquid broth (10 liters) containing glucose (3%), polypeptone (0.5%),

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yeast extract (0.2%), potassium dihydrogenphosphate (0.1%) and magnesium sulfate (0.05%). After 40 d, the culture broth was filtered to provide broth and mycelia. The filtered broth was extracted with EtOAc, and the extract (5.8 g) was chromatographed on silica gel (Wakogel C300, 150 g) with EtOAc/hexane (450 ml each), using a 10% stepwise elution gradient.

The 30% EtOAc/hexane eluate (21.3 mg) was purified in a silica gel column (Wakogel C300, 4 g) with 10% (10 ml  $\times$  5), 15% (10 ml  $\times$  5) and 20% EtOAc/hexane (5 ml  $\times$  10) giving 20 fractions. Fractions 14–16 were combined (7.7 mg) and purified by HPLC (ODS-UG-5 column, Nomura Chemical; flow rate, 2.0 ml/min; eluent, 60% methanol/water). The fraction which eluted at  $t_R$  12.1 min was collected to give **2** (3.2 mg) as a colorless oil. Fraction no. 6 (12.5 mg) was purified by HPLC (ODS-UG-5 column; flow rate, 2.0 ml/min; eluent, 50% methanol/water). The fraction that eluted at  $t_R$  19.8 min was collected to give **3** (4.3 mg) as a colorless oil.

**6-Hydroxy-2,2-dimethyl-2H-chromene (2).** The spectral data for **2** are consistent with 6-hydroxy-2,2-dimethyl-2H-chromene which had previously been isolated from *Aplidium californicum* by Cotellet *et al.*<sup>6)</sup>

**6-Hydroxy-5,7-dimethoxy-2,2-dimethyl-2H-chromene (3).** IR (KBr)  $\nu_{\max}$   $\text{cm}^{-1}$ : 3446, 1490, 1203, 912, 853;  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR: see Table 1.

The 50% EtOAc/hexane (853 mg) fraction was purified in a silica gel column (Wakogel C300, 35 g) with 15% (20 ml  $\times$  10), 20% (20 ml  $\times$  5), 25% (20 ml  $\times$  5), 30% (20 ml  $\times$  5) and 40% acetone/hexane (20 ml  $\times$  5), giving thirty fractions. Fractions 24–30 were combined (14.5 mg) and purified by HPLC (ODS-UG-5 column; flow rate, 2.0 ml/min; eluent, 30% methanol/water). The fraction that eluted at  $t_R$  15.6 min was collected and provided **4** (1.5 mg) as a colorless oil.

**6-Hydroxy-2-hydroxymethyl-5-methoxy-2-methyl-2H-chromene (4).**  $[\alpha]_D^{21} = -2.6^\circ$  ( $c$  0.20, EtOH); IR (KBr)  $\nu_{\max}$   $\text{cm}^{-1}$ : 3446, 2923, 2852, 2360, 1477, 1248, 1058, 952, 867;  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR: see Table 1.

#### Synthesis of **1a**, **1b**, **1c** and ( $\pm$ )-**1**.

**(2R)-2-Hydroxymethyl-6-methoxy-2-methyl-2H-chromene (1a).** **1a** was synthesized from **1** as previously described.<sup>5)</sup>

**(2R)-6-Hydroxy-2-hydroxymethyl-2-methyl-chroman (1b).** To a solution of **1** (21 mg, 0.11 mmol) in EtOH (5.0 ml) was added 10% palladium on carbon (0.1 mg, 0.01 mmol) under a  $\text{H}_2$  atmosphere. The mixture was stirred for 8 h, filtered and then concentrated. The residue was purified by silica gel column chromatography (hexane:EtOAc = 1:1) to give **1b** (21 mg, 0.11 mmol, 100%) as a colorless oil,  $[\alpha]_D^{19} = -9.9^\circ$  ( $c$  0.21, acetone). IR (film)  $\nu_{\max}$   $\text{cm}^{-1}$ : 3353, 2930, 1494, 1454, 1222, 1050, 809;  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ )  $\delta_{\text{H}}$ : 1.24 (3H, s), 1.67 (1H, ddd,  $J = 4.5, 6.2, 14.0$  Hz), 1.99 (1H, ddd,  $J = 6.2, 11.0, 14.0$  Hz), 2.27 (OH, br. s), 2.68 (1H, dt,  $J = 5.2, 17.0$  Hz), 2.79 (1H, ddd,  $J = 6.2, 11.0, 17.0$  Hz), 3.60 (1H, d,  $J = 12.0$  Hz), 3.64 (1H, d,  $J = 12.0$  Hz), 5.40 (OH, br. s), 6.56 (1H, d,  $J = 2.9$  Hz), 6.59 (1H, dd,  $J = 2.9, 8.7$  Hz), 6.66 (1H, d,  $J = 8.7$  Hz);  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ )  $\delta_{\text{C}}$ : 20.4, 21.8, 27.5, 69.1, 76.2, 114.6, 115.4, 117.7, 121.9, 147.1, 149.0. HREIMS  $m/z$  ( $\text{M}^+$ ): Calcd. for  $\text{C}_{11}\text{H}_{14}\text{O}_3$ : 194.0943. Found: 194.0949.

**(2R)-6-Hydroxy-2-hydroxymethyl-2,5,7,8-tetramethyl-chroman (1c).** To a solution of (*R*)-trolox (50 mg, 0.20 mmol) in THF (5.0 ml) was added dropwise  $\text{BH}_3 \cdot \text{SMe}_2$  in THF (a 10 M solution, 0.10 ml, 1.0 mmol) at  $0^\circ\text{C}$ . The mixture was stirred for 10 min at that temperature, and then diluted with water. The mixture was extracted with EtOAc, washed with brine, filtered and concentrated. The residue was purified by silica gel column chromatography (hexane:EtOAc = 1:1) to give compound **1c** (43 mg, 0.18 mmol, 90%) as a colorless solid,  $[\alpha]_D^{19} = -1.1^\circ$  ( $c$  0.10,  $\text{CHCl}_3$ ). IR (film)  $\nu_{\max}$   $\text{cm}^{-1}$ : 3388, 2929, 1454, 1419, 1257, 1086, 1047;  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ )  $\delta_{\text{H}}$ : 1.22 (3H, s), 1.73 (1H, dt,  $J = 13.5, 6.0$  Hz), 1.93 (OH, br. s), 2.00 (1H, m), 2.11 (3H, s), 2.12 (3H, s), 2.16 (3H, s), 2.68 (2H, m), 3.62 (2H, m), 4.28 (OH, br. d);  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ )  $\delta_{\text{C}}$ : 11.3, 11.8, 12.2, 20.3, 20.4, 27.9, 69.4, 75.1, 117.3, 118.7, 121.3, 122.6, 144.9, 145.1. HREIMS  $m/z$  ( $\text{M}^+$ ): Calcd. for  $\text{C}_{14}\text{H}_{20}\text{O}_3$ : 236.1412. Found: 236.1417.

**( $\pm$ )-6-Hydroxy-2-hydroxymethyl-2-methyl-2H-chromene (( $\pm$ )-**1**).** ( $\pm$ )-**1** was synthesized from 2,5-dihydroxybenzaldehyde as previously described.<sup>11)</sup>

**DPPH radical scavenging test.** The DPPH radical scavenging activities were evaluated as previously described.<sup>5)</sup>

**Tyrosinase inhibitory test.** The tyrosinase inhibitory activities were determined by using L-tyrosine or L-dopa as the substrate, essentially as previously described.<sup>12)</sup> The tyrosinase inhibitory test using L-tyrosine as the substrate was conducted as follows: L-tyrosine (400  $\mu\text{l}$  of 5 mM) and 1460  $\mu\text{l}$  of a 50 mM phosphate buffer (pH 6.8) were mixed with 100  $\mu\text{l}$  of a sample in dimethyl sulfoxide. After adding 40  $\mu\text{l}$  of an aqueous solution of mushroom tyrosinase (1000 U/ml, Sigma Chemical Co.), the mixture was incubated at  $37^\circ\text{C}$  for 60 min, and then the absorbance at 475 nm was measured to estimate the activity. Tyrosinase inhibition was calculated as inhibition (%) =  $100 \times (A - B)/A$ , where A is the absorbance of the control, and B is the difference in absorbance before and after incubating the test sample. The tyrosinase inhibitory test using L-dopa as the substrate was conducted as follows: L-dopa (400  $\mu\text{l}$  of 5 mM) and 1480  $\mu\text{l}$  of a 50 mM phosphate buffer (pH 6.8) were mixed with 100  $\mu\text{l}$  of a sample in dimethyl sulfoxide. After adding 20  $\mu\text{l}$  of an aqueous solution of mushroom tyrosinase (1000 U/ml), the mixture was incubated at  $25^\circ\text{C}$  for 30 min, and then the absorbance at 475 nm was measured to estimate the activity. Tyrosinase inhibition was calculated as inhibition (%) =  $100 \times (A - B)/A$ , where A is the absorbance of the control, and B is the difference in absorbance before and after incubating the test sample. The extent of inhibition by the addition of the sample is expressed as the percentage necessary for 50% inhibition ( $\text{IC}_{50}$ ). The Michaelis constant ( $K_m$ ), maximum velocity ( $V_{\max}$ ) and inhibition constant ( $K_i$ ) for tyrosinase were determined by Lineweaver-Burk plots, using various concentrations of L-tyrosine.

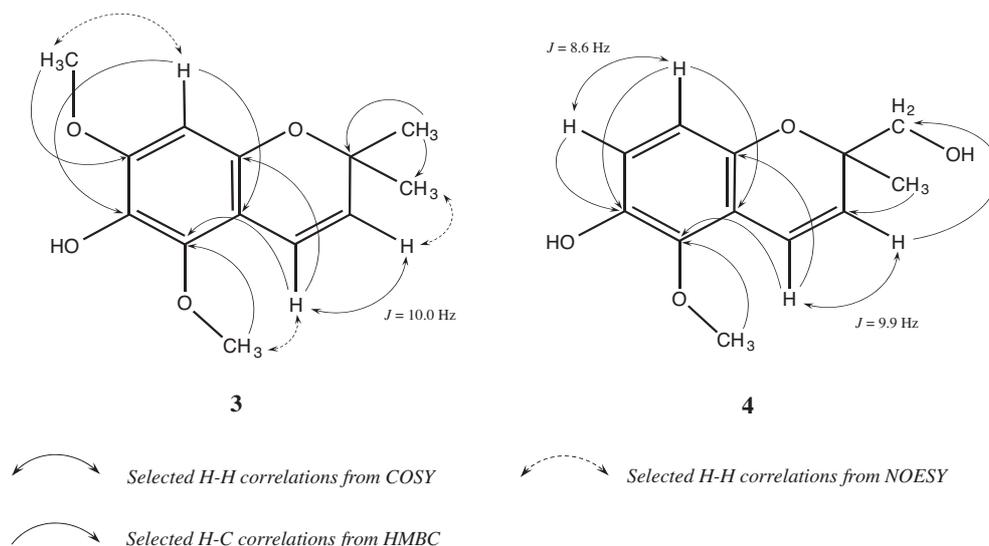
**Melanogenesis inhibitory assay using a three-dimensional human skin model.** The melanogenesis inhibitory assay using a three-dimensional human skin model was performed as previously described, with slight modifications.<sup>13)</sup> The three-dimensional cultured human skin model was placed in 6-well plates and incubated with 5 ml of LLMM at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ . An aqueous solution (70  $\mu\text{l}$ ) of a test sample was applied to the surface of the tissue. The tissue was incubated for 14 d, with LLMM being replaced by a fresh medium on alternate days. After cultivation, the tissue was washed twice with Dulbecco's phosphate-buffered saline (DPBS) to remove the aqueous contaminants, and the melanin content and viability of the tissue cells were measured. To measure the melanin content, the tissue cells were rendered soluble by incubating with 400  $\mu\text{l}$  of aqueous 1 N NaOH at  $80^\circ\text{C}$  for 2 h. The solution was centrifuged at  $1000 \times g$  for 10 min, filtered, and the absorption of the supernatant was determined at 470 nm. The melanin content of the solution was calculated by comparing with the absorbance of synthetic melanin. Cell viability of the human skin model was evaluated by an MTT assay. After cultivating for 14 d, the tissue was placed in a 24-well plate, 3 ml of an MTT solution (MTT diluted with LLMM at 1 mg/ml) was added to each well, and the tissue was incubated at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ . After 3 h of incubation, the tissue was washed twice with DPBS. Two milliliters of the MTT extract, isopropyl alcohol containing 40 mM HCl, and 40  $\mu\text{l}$  of sodium dodecyl sulfate were then added to each well, and the plate was gently shaken at room temperature for 2 h. The absorbance of the extract was measured at 570 nm.

## Result and Discussion

### Structure of the isolated compounds **2–4**

Compound **2** was identified as 6-hydroxy-2,2-dimethyl-2H-chromene by comparing the spectral data for **2** with the reference data.<sup>6)</sup>

High-resolution EIMS of **3** showed a molecular ion peak at  $m/z$  236.1047, consistent with the molecular formula  $\text{C}_{13}\text{H}_{16}\text{O}_4$  (Calcd.: 236.1049). The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra resembled those of **2**. Comparison of the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data for **3** and **2** revealed that **3** differed from **2** by the presence of two methoxy groups ( $\delta_{\text{C}}$  61.3,  $\delta_{\text{H}}$  3.87,  $\delta_{\text{C}}$  56.2,  $\delta_{\text{H}}$  3.84). The positions of the methoxy groups at C-5 and C-7 were determined on the basis of NOESY and HMBC correlations (Fig. 1). Thus, **3** was elucidated as 6-hydroxy-5,7-dimethoxy-2,2-dimethyl-2H-chromene.



**Fig. 1.** Selected H–H and C–H Correlations from COSY, HMBC and NOESY Analyses of Compounds **3** and **4**.

**Table 1.**  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  Data for **3** and **4**

	<b>3</b>		<b>4</b>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$
2	75.7 C		78.6 C	
3	128.1 CH	5.51 d ( $J = 10.0\text{ Hz}$ )	127.8 CH	5.67 d ( $J = 9.9\text{ Hz}$ )
4	116.9 CH	6.53 d ( $J = 10.0\text{ Hz}$ )	119.2 CH	6.69 d ( $J = 9.9\text{ Hz}$ )
4a	108.2 C		114.6 C	
5	142.7 C		142.9 C	
6	132.3 C		145.9 C	
7	147.6 C		115.2 CH	6.73 d ( $J = 8.6\text{ Hz}$ )
8	96.2 CH	6.23 s	112.3 CH	6.51 d ( $J = 8.6\text{ Hz}$ )
8a	146.1 C		142.7 C	
9	27.5 $\text{CH}_3$	1.40 s	68.4 $\text{CH}_2$	3.60 d ( $J = 11.5\text{ Hz}$ ) 3.68 d ( $J = 11.5\text{ Hz}$ )
10	27.5 $\text{CH}_3$	1.40 s	22.0 $\text{CH}_3$	1.34 s
	56.2 $\text{OCH}_3$	3.84 s	62.3 $\text{OCH}_3$	3.81 s
	61.3 $\text{OCH}_3$	3.87 s		

High-resolution EIMS of **4** showed a molecular ion peak at  $m/z$  222.0890, consistent with the molecular formula  $\text{C}_{12}\text{H}_{14}\text{O}_4$  (Calcd.: 222.0892). The IR spectrum of **4** showed absorption bands for OH groups ( $3446\text{ cm}^{-1}$ ) and a benzene ring ( $867\text{ cm}^{-1}$ ). As shown in Table 1, the  $^1\text{H-}$  and  $^{13}\text{C-NMR}$  data for **4** were similar to those for **1**, except for the presence of a methoxy group ( $\delta_{\text{C}}$  62.3,  $\delta_{\text{H}}$  3.81) at position 5. We thus concluded that **4** was 5-methoxy-daedalin A; this was substantiated by C–H correlations between the methoxy proton ( $\delta_{\text{H}}$  3.81) and C-5 ( $\delta_{\text{C}}$  142.9), and between C-5 and H-4 ( $\delta_{\text{H}}$  6.69) in the HMBC spectrum (Fig. 1).

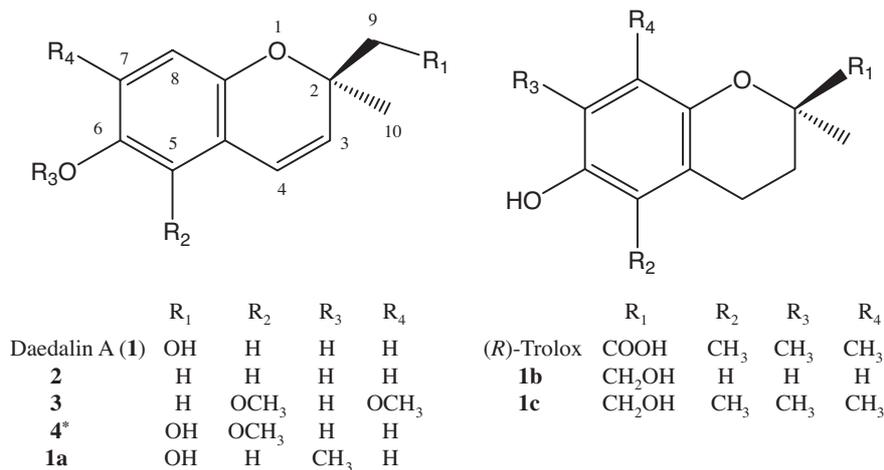
#### Tyrosinase inhibitory activities

As shown in Fig. 3, a hydroxyl and carboxyl group of L-tyrosine could adopt conformations similar to that of the two hydroxyl groups of **1**. Compound **1** potently suppressed the hydroxylation of L-tyrosine by tyrosinase at low concentrations, but did not suppress the oxidation

of L-dopa by tyrosinase at a concentration of  $800\text{ }\mu\text{M}$ . Based on these results, the tyrosinase inhibitory activity of **1** was probably caused by competitive inhibition with L-tyrosine. This was confirmed by Lineweaver-Burk plots, using L-tyrosine as a substrate for tyrosinase with **1** (Fig. 4). Compound **1** at  $50\text{ }\mu\text{M}$  and  $100\text{ }\mu\text{M}$  exhibited the same  $V_{\text{max}}$  value of  $1.5 \times 10^{-2}$  and  $K_m$  values of  $7.5 \times 10^{-1} \pm 4.6 \times 10^{-2}$  ( $n = 3$ ) and  $1.0 \pm 4.2 \times 10^{-2}$  ( $n = 3$ ) mM. Therefore, daedalin A was proved to be a competitive inhibitor of L-tyrosine hydroxylation by tyrosinase, with a  $K_i$  value of  $4.6 \times 10 \pm 9.3 \times 10^{-1}$  ( $n = 3$ )  $\mu\text{M}$ .

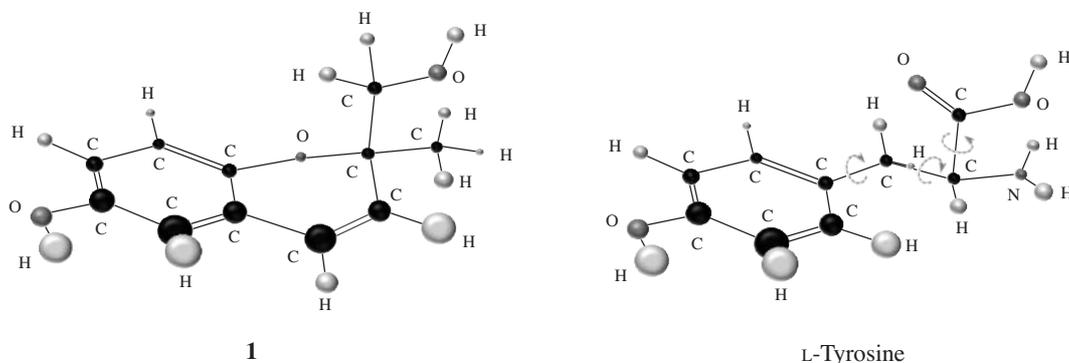
The tyrosinase inhibitory activities of the isolated compounds (**1–4**) and synthesized compounds (**1a–1c**, and ( $\pm$ )-**1**) are shown in Table 2. Compound **1** inhibited the tyrosinase activity by up to 60.7% at  $400\text{ }\mu\text{M}$ , whereas 6-methoxy-daedalin A (**1a**) and 9-deoxy-daedalin A (**2**) did not show activity at  $400\text{ }\mu\text{M}$ . These results suggest that the hydroxyl groups at positions 6 and 9 might have played an important role in the tyrosinase inhibitory activity of chromans. Interestingly, a racemer of **1** (( $\pm$ )-**1**) had weaker activity ( $\text{IC}_{50}$ ,  $289\text{ }\mu\text{M}$ ) than **1** ( $\text{IC}_{50}$ ,  $194\text{ }\mu\text{M}$ ). These results also support the competitive inhibitory mechanism for **1** on tyrosinase which was elucidated by the kinetic study of **1** and L-tyrosine.

Yu has reported the tyrosinase inhibitory effect of (*R*)-trolox bearing methyls at positions 5, 7 and 8.<sup>14</sup> However, the activity was extremely weak ( $\text{IC}_{50}$ ,  $6100\text{ }\mu\text{M}$ ), about 30 times lower than that of **1**. Compound **1c** did not show any activity at  $800\text{ }\mu\text{M}$ , whereas the activity of **1b** was stronger ( $\text{IC}_{50}$ ,  $345\text{ }\mu\text{M}$ ) than that of arbutin. Structurally, **1b** and **1c** differ by the presence of methyl groups at positions 5, 7 and 8. In addition, **4**, which has a methoxy group at position 5, did not show activity at a concentration of  $400\text{ }\mu\text{M}$ , and **3**, which was substituted with methoxy groups at position 5 and 7, did not show activity at  $800\text{ }\mu\text{M}$ . Therefore, the tyrosinase inhibitory activities of the chroman analogs were substantially decreased by methyl and methoxy groups on the benzene ring, possibly due to restricted binding to the enzyme. A tyrosinase inhibitory test revealed compound **1** to show the strongest activity among our tested chroman analogs.



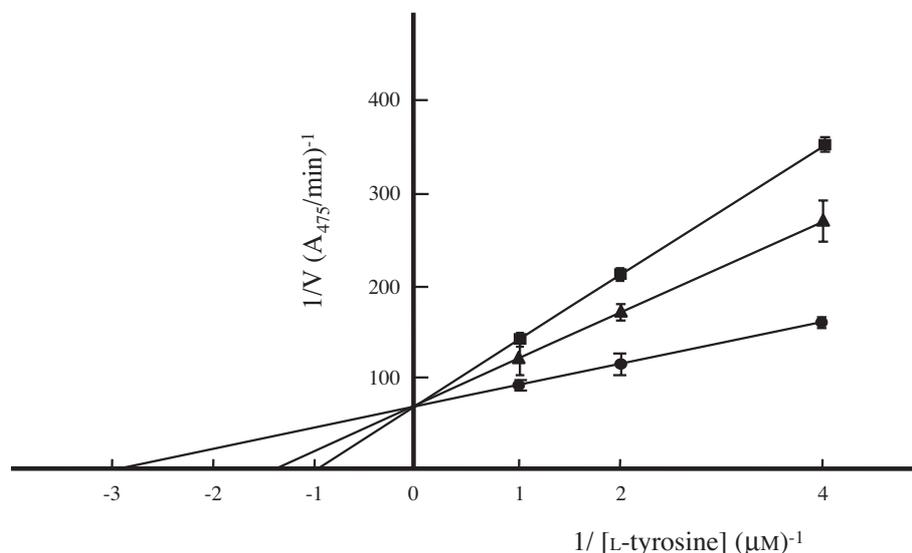
**Fig. 2.** Structures of Compounds **1-4**, **1a-1c**, and (*R*)-Trolox.

\*Possible absolute structure which might be different.



**Fig. 3.** Optimum Conformation of Compound **1** and Selected Conformation of L-Tyrosine.

The optimum conformation of compound **1** was generated by using MM2 computations of the Chem 3D program.



**Fig. 4.** Lineweaver-Burk Plots of Mushroom Tyrosinase and L-Tyrosine without (●), and with 50 μM (▲) and 100 μM (■) of **1**.

#### DPPH radical scavenging activities

Daedalin A (**1**) showed potent free radical scavenging activity.<sup>5)</sup> We compared the DPPH radical scavenging activity of **1** with that of related compounds. As shown in Table 3, at 100 μM, all the tested compounds except **1a** scavenged more than 50% of the DPPH radicals. Among the compounds, **1** (IC<sub>50</sub>, 16.9 μM), **1b** (IC<sub>50</sub>,

17.8 μM) and **1c** (IC<sub>50</sub>, 14.0 μM) showed strong activities, comparable to that of (*R*)-trolox (IC<sub>50</sub>, 13.9 μM) and α-tocopherol (IC<sub>50</sub>, 14.2 μM). However **1a**, which lacked a phenolic hydroxyl group, showed no activity at 500 μM. Therefore, the phenolic hydroxyl group was essential for the antioxidant activity of our tested compounds.

Melanin is also biosynthesized by the auto-oxidative

**Table 2.** Tyrosinase Inhibitory Activities of **1–4**, (**±**)-**1**, **1a–1c**, Arbutin and (*R*)-Trolox

Compound	Inhibition $\pm$ SD (%) <sup>a</sup>				IC <sub>50</sub> ( $\mu$ M)
	100 $\mu$ M	200 $\mu$ M	400 $\mu$ M	800 $\mu$ M	
Daedalin A ( <b>1</b> )	34.9 $\pm$ 1.1	50.9 $\pm$ 6.7	60.7 $\pm$ 3.2	NT <sup>b</sup>	194
( <b>±</b> )- <b>1</b>	23.7 $\pm$ 5.7	45.5 $\pm$ 3.1	55.6 $\pm$ 1.1	NT	289
<b>2</b>	NI <sup>c</sup>	NI	NI	NT	>400
<b>3</b>	NT	NT	NT	NI	>800
<b>4</b>	NI	NI	NI	NT	>400
<b>1a</b>	NI	NI	NI	NI	>800
<b>1b</b>	15.9 $\pm$ 1.9	28.5 $\pm$ 3.5	58.2 $\pm$ 3.7	NT	345
<b>1c</b>	NI	NI	NI	NI	>800
Arbutin	22.0 $\pm$ 4.1	28.6 $\pm$ 9.5	34.7 $\pm$ 5.0	51.0 $\pm$ 3.1	672
( <i>R</i> )-Trolox	NI	NI	NI	8.6 $\pm$ 2.9	6100

<sup>a</sup>Each value is expressed as the means  $\pm$  SD from three tests.<sup>b</sup>NT, not tested<sup>c</sup>NI, no inhibition**Table 3.** DPPH Radical Scavenging Activities of **1–4**, (**±**)-**1**, **1a–1c**,  $\alpha$ -Tocopherol and (*R*)-Trolox

Compound	Inhibition $\pm$ SD (%) <sup>a</sup>				IC <sub>50</sub> ( $\mu$ M)
	20 $\mu$ M	40 $\mu$ M	50 $\mu$ M	100 $\mu$ M	
Daedalin A ( <b>1</b> )	57.4 $\pm$ 2.5	NT <sup>b</sup>	79.9 $\pm$ 0.8	83.8 $\pm$ 0.2	16.9
( <b>±</b> )- <b>1</b>	44.6 $\pm$ 0.8	72.2 $\pm$ 0.5	NT	84.9 $\pm$ 0.9	23.9
<b>2</b>	17.4 $\pm$ 1.6	NT	35.3 $\pm$ 0.4	57.0 $\pm$ 0.1	83.8
<b>3</b>	18.0 $\pm$ 0.1	NT	44.5 $\pm$ 1.0	81.7 $\pm$ 0.1	57.3
<b>4</b>	32.4 $\pm$ 1.4	64.0 $\pm$ 0.6	NT	81.2 $\pm$ 0.2	31.1
<b>1a</b>	0.4 $\pm$ 2.0	NT	1.2 $\pm$ 2.4	1.9 $\pm$ 0.6	>500
<b>1b</b>	54.0 $\pm$ 1.8	NT	79.5 $\pm$ 0.6	83.9 $\pm$ 0.6	17.8
<b>1c</b>	70.2 $\pm$ 0.7	NT	87.5 $\pm$ 0.3	87.9 $\pm$ 0.1	14.0
$\alpha$ -Tocopherol	72.2 $\pm$ 0.4	NT	92.8 $\pm$ 2.2	94.6 $\pm$ 0.7	14.2
( <i>R</i> )-Trolox	69.8 $\pm$ 1.5	85.9 $\pm$ 0.3	84.0 $\pm$ 0.3	84.0 $\pm$ 0.4	13.9

<sup>a</sup>Each value is expressed as the means  $\pm$  SD from three tests.<sup>b</sup>NT, not tested

action of dopaquinone, following the oxidation of L-tyrosine to L-dopa and of L-dopa to dopaquinone by tyrosinase.<sup>15)</sup> It has been reported that some antioxidants prevent melanogenesis. For example, Hayakawa *et al.* have reported that an oral intake of  $\alpha$ -tocopherol was an effective treatment for facial hyperpigmentation,<sup>16)</sup> and Matsuda *et al.* have reported that an antioxidant extract from *Myrica rubra* inhibited melanin synthesis.<sup>17)</sup> In addition to the tyrosinase inhibitory activity of **1**, the antioxidant activity might enhance the inhibitory activity toward melanin synthesis.

#### Melanogenesis inhibitory activity of **1**

We evaluated the melanogenesis inhibitory activity of **1** on a three-dimensional cultured human skin model composed of normal human melanocytes and keratinocytes, as well as human skin. As shown in Fig. 5A, after 14 d of incubation of the cultured human skin model with **1**, the pigmentation of the model was dose-dependently suppressed. Figure 5B demonstrates that melanin synthesis by the model was reduced to 72%  $\pm$  4.2 (n = 3) and 49%  $\pm$  2.3 (n = 3) by **1** at 1.4 and 2.8  $\mu$ mol per well, respectively. In contrast, the inhibitory activity of arbutin was 67%  $\pm$  4.1 (n = 3) at 2.8  $\mu$ mol per well. The cell viability of the human skin model was determined by using an MTT assay. After 14 d incubation with 2.8  $\mu$ mol of **1** per well, the cell viability did not change (102%  $\pm$  7.5, n = 3). Thus, **1** inhibited melanin biosynthesis in the human skin model without affecting the cell viability.

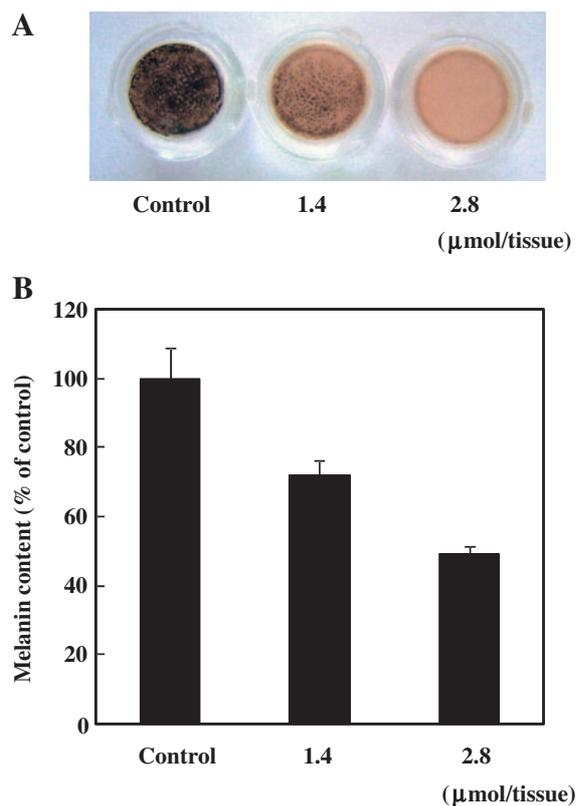
## Conclusion

This study shows that daedalin A had the most potent tyrosinase inhibitory activity of the compounds tested. An analysis of structure-activity relationships and Lineweaver-Burk plots confirmed that the tyrosinase inhibitory activity of daedalin A was due to competitive inhibition with L-tyrosine.

Furthermore, daedalin A inhibited the pigmentation of a three-dimensional cultured human skin model without affecting the cell viability. In the epidermis, dermal pigmentation appears after the synthesis of melanin in melanosomes and the transfer of melanin pigment to epidermal cells. Melanin transfer is mediated by the expression of protease-activated receptor 2 (PAR-2) in keratinocytes, but not in melanocytes.<sup>18,19)</sup>

Paine *et al.* have reported that a soybean extract inhibited pigmentation in a three-dimensional cultured human skin model, although the extract did not show tyrosinase inhibitory activity,<sup>20)</sup> indicating that the inhibition of pigmentation was caused by the inhibition of PAR-2 activation. Since daedalin A inhibited melanin synthesis as well as tyrosinase activity, its inhibitory effect on pigmentation was probably due to the direct inhibition of tyrosinase activity in melanocytes. In addition to the tyrosinase inhibitory activity, the antioxidant activity might also have contributed to the melanogenesis inhibitory effect of daedalin A.

Yoon *et al.* have reported the melanogenesis inhibitory test using a three-dimensional cultured human skin



**Fig. 5.** Inhibitory Effect of Melanogenesis by **1** in a Three-Dimensional Human Skin Model.

A, Three-dimensional cultured human skin model without or with 1.4  $\mu\text{mol}$  and 2.8  $\mu\text{mol}$  of **1**. B, Melanin content of a three-dimensional cultured human skin model without or with 1.4  $\mu\text{mol}$  and 2.8  $\mu\text{mol}$  of **1**. The melanin content of the model was quantitatively estimated. Each value is presented as the mean  $\pm$  SD from three separate experiments.

model to be a reliable alternative to animal tests for evaluating dermal hyperpigmentation.<sup>21)</sup> Studying melanin synthesis by using this model, Majmudar *et al.* have reported the inhibitory effect of kojic acid,<sup>22)</sup> and Sugimoto *et al.* have reported the inhibitory effect of  $\alpha$ -arbutin.<sup>13)</sup> The melanogenesis inhibitory activity of daedalin A on the three-dimensional cultured human skin model was stronger than that of arbutin. Thus, daedalin A is likely to inhibit melanin synthesis in human skin and could be used as a pharmaceutical constituent for preventing dermal hyperpigmentation.

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