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N-[(Dihydroxyphenyl)acyl]serotonins as potent inhibitors of tyrosinase from mouse and human melanoma cells

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ABSTRACT

A series of *N*-acyl derivatives of tyramine, tryptamine, and serotonin were synthesized and tested on anti-melanogenic activity. The serotonin derivatives such as *N*-caffeoylserotonin (**3**) and *N*-protocatechuoylserotonin (**9**) were inhibitory to tyrosinase from mouse B16 and human HMV-II melanoma cells, while the corresponding derivatives of tryptamine and 5-methoxytryptamine were almost inactive or less active than the serotonin derivatives. The inhibitory activity of the serotonin derivatives increased with increasing number of phenolic hydroxyl groups in the acyl moiety. Melanin formation in the culture of B16 cells was suppressed by **3** and **9** with no cytotoxicity in the concentration range tested (IC_{50} = 15, 3 and 111 μM for **3**, **9**, and kojic acid, respectively). Thus the *N*-acylserotonin derivatives having a dihydroxyphenyl group are potential anti-melanogenic agents. Their inhibition of tyrosinase is primarily performed through the 5-hydroxyindole moiety and further strengthened by the phenolic hydroxyl groups in the acyl moiety.

Tyrosinase inhibitors are important agents for treatment of skin hyperpigmentation due to sun burning or aging, since the pigment, melanin, is formed in a multistep process whose key steps are catalyzed by the copper-containing enzyme tyrosinase (EC 1.14.18.1).[1,2] Most of the inhibitors of tyrosinase are, therefore, copper chelators[3] or phenolic compounds structurally analogous to the substrates, L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA).[4–6] Recently, polyhydroxycinnamamides[7] and benzamides[8] were reported as a new class of potent tyrosinase inhibitors, but their structures were rather limited to ω-phenylalkylamides. Heterocyclic ring-containing alkylamides with anti-melanogenic activity are rare except for *N*-coumaroyl- and *N*-feruloylserotonin in safflower seeds.[9] Although these structures are interesting with regard to the indole intermediates in melanin biosynthesis,[2] little is known about the effect of the indole moiety on the anti-melanogenic activity. Thus, we synthesized a series of *N*-acyl derivatives of tryptamine, serotonin, and tyramine using caffeic, ferulic, vanillic, protocatechuic, hydroxybenzoic, and benzoic acids to study the structure–activity relationship (Fig. 1).

The synthesis was done by the acid chloride method, in which the phenolic hydroxyl groups were protected by acetylation and, after coupling with the amines, they were deprotected with hydrazine hydrate.[10]

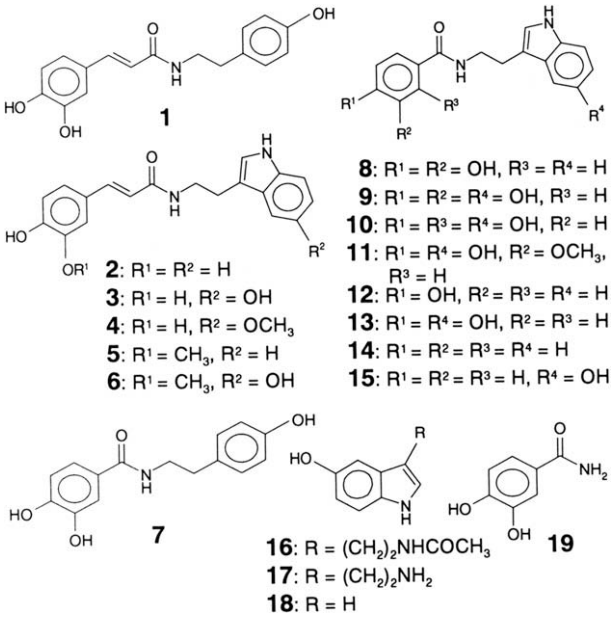

Figure 1. Structures of the compounds.

The anti-melanogenic activity was tested on the inhibition of the catecholase activity of tyrosinase extracted from mouse B16

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Table 1
Anti-melanogenic activities of the synthesized compounds

Compound[a]			Mouse B16 melanoma cells				Human HMV-II melanoma cell tyrosinase
			Tyrosinase inhibition		Melanin inhibition	Viability	
No.	*A*	*N*	IC_{50}[b] (μM)	% at 100 μM[b]	IC_{50}[b] (μM)	% at 20 μM[c]	% at 100 μM[b]
1	C	Y	—	15	ne	84**	30 ± 6
2	C	T	—	2	ne	54**	14 ± 4
3	C	S	28 ± 11	73	15	99	55 ± 10
4	C	M	—	1	ne	41**	4 ± 3
5	F	T	—	4	>30	102	13 ± 7
6	F	S	216 ± 10	29	23	100	48 ± 5
7	P	Y	—	6	ne	88**	9 ± 2
8	P	T	—	2	ne	69**	7 ± 5
9	P	S	73 ± 9	48	3	99	43 ± 9
10	D	S	42 ± 13	50	15	101	39 ± 9
11	V	S	211 ± 13	32	>30	93	16 ± 6
12	H	T	—	0	>30	94	0 ± 3
13	H	S	233 ± 32	31	>30	100	9 ± 5
14	B	T	—	0	ne	85**	1 ± 1
15	B	S	333 ± 29	22	>30	96	23 ± 5
16	A	S	311 ± 13	23	>30	93*	18 ± 3
17	S		550 ± 93	22	>30	101	15 ± 7
18	5HI		68 ± 3	62	13	100	54 ± 2
Kojic acid			61 ± 11	55	111	99[d]	52 ± 5

[a] *A* and *N* represent the acyl and amine moieties, respectively: C = caffeoyl, F = feruloyl, P = protocatechuoyl, D = 2,4-dihydroxybenzoyl, V = vanilloyl, H = *p*-hydroxybenzoyl, B = benzoyl, A = acetyl, Y = tyramine, T = tryptamine, S = serotonin, M = 5-methoxytryptamine, and 5HI = 5-hydroxyindole.
[b] Values are means or means ± sd (standard deviation), $n = 3–5$. '—' = too large to be determined. ne = not evaluated for cytotoxicity.
[c] Values are means of 8 or 9 wells. ** and * represent $p < 0.01$ and $p < 0.05$ versus control by Student's *t*-test, respectively.
[d] At 200 μM.

melanoma cells using L-DOPA as the substrate.[11] Percent inhibition for each compound was calculated from the enzyme activity in the presence of the compound at a concentration of 100 μM (Table 1). A noteworthy fact was that all *N*-acylserotonin derivatives (**3**, **6**, **9**–**11**, **13**, **15**, and **16**) inhibited the tyrosinase, while the tryptamine derivatives (**2**, **5**, **8**, **12**, and **14**) were inactive or weakly active inhibitors under the same experimental condition. Furthermore, serotonin (**17**) and 5-hydroxyindole (**18**) were active and a 5-methoxyindole derivative (**4**) was almost inactive in this assay.[12] These results indicate that the 5-hydroxyl group in the indole moiety plays an essential role in inhibiting tyrosinase. The function of this hydroxyl group needs the indole ring, since the tyramine derivatives (**1** and **7**) were less active than the corresponding serotonin derivatives (**3** and **9**) (Table 1) and 5-hydroxyindan and 6-hydroxyquinoline were slightly active and inactive, respectively (data not shown). The inhibitory activity was also affected by the structure of the acyl moiety. The IC_{50} of the *N*-acylserotonins decreases in the order of **15** > **13** ⩾ **6** ⩾ **11** > **9** ⩾ **10** > **3**, that is, the order of increasing number of their hydroxyl groups. This correlation implies that the inhibitory activity is strengthened by the phenolic hydroxyl group(s) in the acyl moiety.

The serotonin derivatives also inhibited human tyrosinase in HMV-II melanoma cells[13] (Table 1). In this case, *N*-caffeoyl- (**2**), *N*-feruloyl- (**5**), and *N*-protocatechuoyltryptamine (**8**) showed weak inhibitory activity, but other tryptamine derivatives were inactive. The tyramine derivatives **1** and **7** were stronger than the tryptamine derivatives **2** and **8**, but weaker than the corresponding serotonin derivatives **3** and **9**, respectively. These facts indicate that the catechol or guaiacol moiety also contributes to the inhibition of human tyrosinase, while they are less effective than the 5-hydroxyindole moiety.

The anti-melanogenic activity was further tested in the mouse B16 melanoma cell culture.[14] Five serotonin derivatives (**3**, **6**, **9**, **10**, and **11**) showed the dose-dependent suppression in the medium darkening due to melanin formation. The IC_{50} values are summarized in Table 1. No cytotoxicity was found with these compounds by the tetrazolium reduction assay at the concentrations below 30 μM.[14] Compounds **5**, **12**, **13**, and **15**–**17** were slightly active or inactive in suppressing the medium darkening, and other compounds (**1**, **2**, **4**, **7**, **8**, and **14**) showed cytotoxicity in the concentration range of 10–30 μM (Table 1) so that their anti-melanogenic activities were not accurately evaluated. Thus, the serotonin derivatives with a catechol or guaiacol moiety were active, the corresponding tryptamine derivatives were cytotoxic, and other simpler compounds (except **18**) were neither cytotoxic nor active in suppressing the cellular melanogenesis.

Among the above extracellular melanin formation inhibitors, *N*-protocatechuoylserotonin (**9**) showed the lowest IC_{50} and was about 40-fold stronger than kojic acid, the usual anti-melanogenic agent. However, as shown in Figure 2A, the intracellular melanin accumulation in B16 cells was not suppressed by this compound in a concentration range of 2–40 μM. In the case of HMV-II cells, compound **9** (20–40 μM) suppressed by 20% the intracellular melanin content, but cytotoxicity was found at the concentration of 40 μM (Fig. 2B). Other serotonin derivatives **3** and **6** were also inactive in suppressing the intracellular melanin accumulation in B16 cells (data not shown).

The inhibition mechanism of **9** with B16 tyrosinase was kinetically studied. *N*-Acetylserotonin (**16**) and protocatechuamide (**19**) were also studied for comparison.[15] The Lineweaver–Burk plots obtained with different concentrations of **9** intersect the *X*-axis at very close points (Fig. 3A) and the same is found for **16** (Fig. 3B), while those for **19** cross on the *Y*-axis (Fig. 3C). These results indicate that **9** and **16** apparently inhibit the enzyme noncompetitively (apparent K_i = 64 μM and 240 μM, respectively) and **19** does competitively (K_i = 3.7 mM) in the L-DOPA oxidation. Next, the enzyme was preincubated with **16** without or with a small amount of L-DOPA. The residual activity of the enzyme preincubated only with **16** was independent of the preincubation, while it decreased with the preincubation time when both **16** and L-DOPA were present in the mixture (Fig. 3D).

Mushroom tyrosinase is widely used for screening of anti-melanogenic agents.[3,4] We tested the effect of the present compounds on mushroom tyrosinase. The initial rates (% of the control, mean ± sd, $n = 3$) were 268 ± 8 for **3**, 191 ± 10 for **9**, and 36 ± 2 for kojic acid at a concentration of 100 μM, showing that **3** and **9** acti-

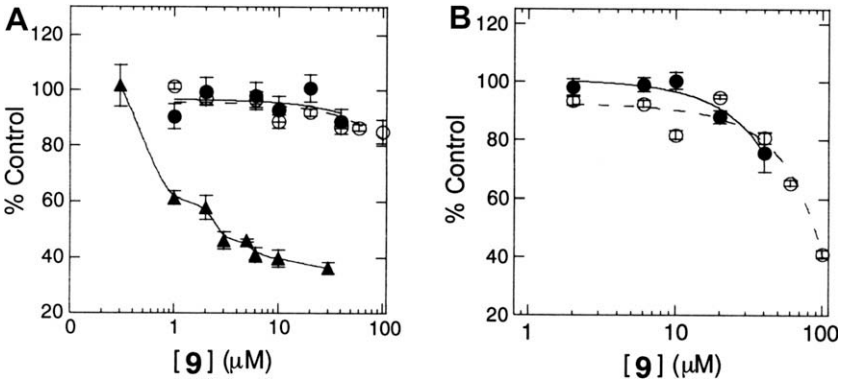

Figure 2. Anti-melanogenic activity and cytotoxicity of compound **9** in cell cultures. (A) Mouse B16 melanoma cells. (B) Human HMV-II melanoma cells. Values (% control) are the means ± SEM (standard error of the mean, $n = 3–4$) in a representative experiment that was repeated with comparable results. ○, cell viability; ▲, extracellular melanin; and ●, intracellular melanin.

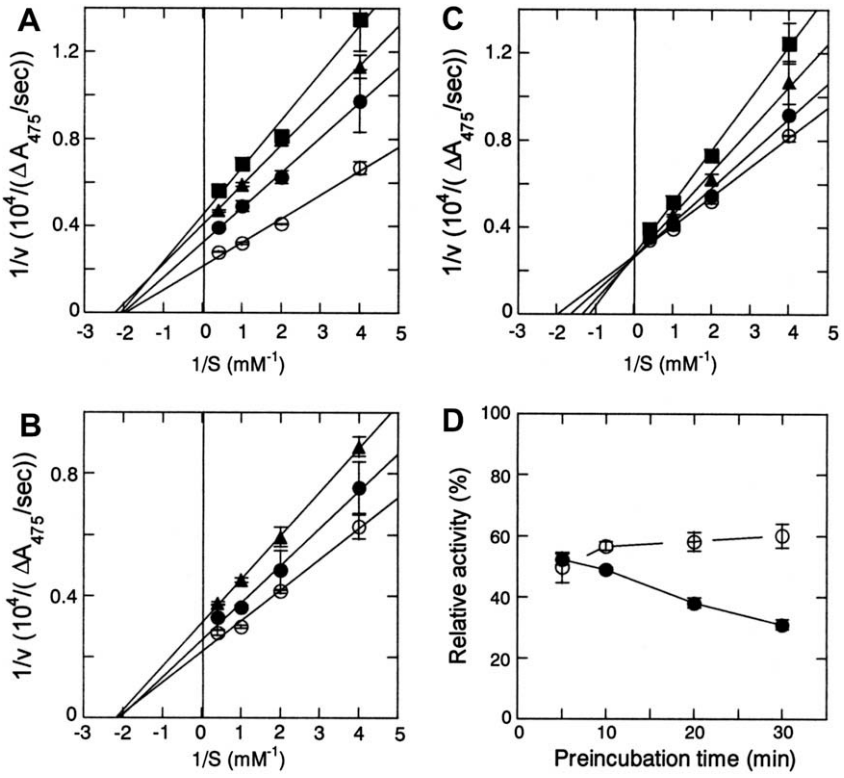

Figure 3. B16 tyrosinase reactions in the presence of compounds **9**, **16**, and **19** with L-DOPA. (A) Lineweaver–Burk (L–B) plots for **9** (0 (○), 30 (●), 50 (▲), and 70 μM (■)); (B) L–B plots for **16** (0 (○), 30 (●), and 100 μM (▲)); and (C) L–B plots for **19** (0 (○), 300 (●), 1000 (▲), and 2500 μM (■)). (D) shows the effect of preincubation of the enzyme with **16** without (○) or with L-DOPA (●). The mixtures prepared by adding 10 μl ethanol containing 20 mM **16** and 50 μl of the enzyme solution into 0.47 ml 50 mM phosphate buffer (pH 6.8) lacking or containing 0.125 mM L-DOPA were preincubated at 37 °C for 5, 10, 20, or 30 min and then mixed with 0.47 ml of the phosphate buffer containing 5.3 mM L-DOPA for the enzyme activity measurement. The control activity (=100) was obtained after 31 min preincubation without **16**. Every point is the mean ± sd ($n = 3$). Plot lines in A, B, and C are drawn by least square linear regression.

vated mushroom tyrosinase in contrast to the strong inhibition of B16 and HMV-II enzymes. Mushroom tyrosinase is reported to be activated by various kinds of compounds.[2] It is not clear how **3** and **9** affect this enzyme, but it should be mentioned that the assay with mushroom tyrosinase might create a misleading expectation for the activity of compounds against mammalian tyrosinases.

N-Feruloylserotonin (**6**) was found to be an tyrosinase inhibitor,[9] and was studied on anti-atherogenic property.[16] The related tryptamine derivative **5** was recently shown to regulate the production of cytokines such as adiponectin[17] and TNF-α.[18] The caffeoyl analogue, **2**,[19] and the benzoyl analogues, **14**[20] and **15**,[21] also appeared in the literature, but none of the present compounds other than **6**, **1**, and **18** has been reported in any study concerning anti-melanogenic activity. The tyramine derivative **1** was the strongest one in the panel for human tyrosinase inhibitors by Okombi et al.,[7] yet the corresponding serotonin derivative (**3**) is stronger than **1**.

The present study has revealed that *N*-acylserotonins and their nucleus, 5-hydroxyindole (**18**), are good inhibitors against mouse and human melanoma tyrosinase, while *N*-acyltryptamines are all poor inhibitors. Among the *N*-acylserotonins, *N*-caffeoylserotonin (**3**) is the strongest inhibitor, and *N*-caffeoyl- (**1**) and *N*-protocatechuoyltyramine (**7**) are moderate or weak inhibitors of the tyrosinases. These facts indicate that the tyrosinase inhibition is

primarily performed by the 5-hydroxyindole or catechol moiety. Tyrosinase contains two copper atoms in a binuclear complex, and this complex is thought to be the catalytic center.[1,22] The catechol hydroxyl groups of the substrate are bound to the copper atoms so that a catechol-containing inhibitor would compete for the copper center with the substrate. The competitive inhibition by protocatechuamide (**19**) is consistent with this mechanism. Thus, the question is why the inhibitory activities of the catechol-containing tryptamine derivatives (**2** and **8**) are so weak as compared with the corresponding serotonin derivatives (**3** and **9**). The 5-hydroxyl group on the indole ring should be capable of binding to the copper atom, as suggested by the UV spectral change of serotonin with copper ions.[23] This expectation is supported by the fact that 5-hydroxyindole (**18**) and serotonin show inhibitory activity. If a compound such as **3** and **9** has both 5-hydroxyindole and catechol moieties, the 5-hydroxyindole moiety would occupy the copper center prior to the catechol group, and the latter would stabilize the whole binding through some interaction with amino acid residue(s) of the enzyme. The catechol group also can bind to the copper center, and in this case 5-hydroxyl group on the indole ring may act to strengthen the binding. However, this orientation (copper binding by the catechol group) would be abortive for the tryptamine derivatives **2** and **8** probably because of a steric hindrance by the indole ring that lacks a hydroxyl group to compensate for the disadvantage in binding energy by an interaction (e.g., hydrogen bond formation) to the enzyme. This role of the hydroxyl group at the opposite site against the copper binding moiety is possibly played by the phenolic groups in other *N*-acylserotonins as well. Therefore, the number of the phenolic hydroxyl group of compounds **3**, **6**, **9**, **10**, **11**, **13**, and **15** may positively correlate with the inhibitory activity. The difference between the activities of the catechol- and resorcinol-containing derivatives (**9** and **10)** suggests that the positions of phenolic hydroxyl groups also affect the inhibition. 5-Hydroxyindole (**18**) itself inhibited B16 tyrosinase as strongly as kojic acid (Table 1), but **3** or **10** was stronger than **18**. This fact indicates that the activity of the nucleus 5-hydroxyindole can be increased by the introduction of a suitable side chain with phenolic groups. The comparison between the serotonin and tyramine derivatives (**1** vs **3**; **7** vs **9**) indicates that the NH group of the indole ring also plays an important role in the present inhibition, presumably by an interaction with the copper center or with the polypeptide around it. Of course, there is another possibility that the serotonin derivatives bind to the enzyme at a site remote from the copper center and thereby inhibit the enzyme via an allosteric effect. The noncompetitive-type inhibition by **9** and **16** might support this possibility. However, noncompetitive inhibition can be caused by another mechanism whereby the inhibitor binds to the enzyme in a form different from those receiving the substrate.[24,25] Tyrosinase takes three forms for copper states in the catalytic cycle, that is, the oxidized (*met*) form, the oxygenated (*oxy*) form, and the reduced (*red*) form. In the catechol oxidation, the substrate binds to the *met* and *oxy* forms. If an inhibitor binds only to the *red* form, the inhibition is apparently noncompetitive with respect to the substrate, as demonstrated by iodide[24] and cyanide ions.[25] The fact that the preincubation of the enzyme with **16** in the presence of L-DOPA enhanced the inhibition time-dependently (Fig. 3D) suggests that the *red* form is involved in this inhibition, because the *red* form is generated only during the catalytic process. However, further study is needed to clarify the inhibitory mechanism.

The *N*-acylserotonins are good inhibitors for the cell-free tyrosinase and melanogenesis in the culture medium. These two inhibitory activities show a similar tendency concerning the structure–activity relationship, but there are some exceptions. For instance, *N*-protocatechuoylserotonin (**9**), which is weaker than *N*-caffeoylserotonin (**3**) for the cell-free enzyme, is very much stronger than **3** in the suppression of culture darkening. However, no compound significantly suppressed the melanin accumulation in B16 cells. To these cells, the serotonin derivatives were not cytotoxic. On the other hand, the corresponding tryptamine derivatives (of the structures with more surface-active property) showed strong cytotoxicity. These facts imply a poor membrane permeability of the polar serotonin derivatives. Improvement of this point is also to be addressed in future.

In conclusion, this study shows that 5-hydroxyindole ring is a promising component for tyrosinase inhibitors in combination with a suitable pendant group such as the catechol-containing acyl group.

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10. 3-(3,4-Dihydroxyphenyl)-*N*-[2-(5-hydroxyindol-3-yl)ethyl]-2-propenamide (**3**) was synthesized by coupling serotonin hydrochloride with 3-(3,4-diacetoxyphenyl)-2-*trans*-propenoyl chloride in dimethylformamide. The reaction product was worked up in the usual way, treated with hydrazine hydrate for deprotection,[17] and purified by silica gel column chromatography (Wako gel C-300). The main product was eluted in 75–100% ethyl acetate/benzene fractions. These fractions were combined, and concentrated to give a residue, which was crystallized from ethyl acetate/hexane as fine needles, 60% yield, mp 138–140 °C and 174–175 °C (with multiple phase transitions), Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.45, H, 5.36, N, 8.28. Found: C, 66.92; H, 5.47; N, 8.00. ^{1}H NMR (270 MHz, acetone-d_6) δ: 2.909 (2H, t, J = 7 Hz, CH_2), 3.591 (2H, m, CH_2), 6.454 (1H, d, J = 15 Hz, *CH*=CH), 6.703 (1H, dd, J = 8 and 2 Hz, H-6′), 6.829 (1H, d, J = 8 Hz, H-5), 6.930 (1H, dd, J = 8 and 2 Hz, H-6), 7.034 (1H, d, J = 2 Hz, H-4′), 7.076 (1H, d, J = 2 Hz, H-2), 7.094 (1H, s, H-2′), 7.193 (1H, d, J = 8 Hz, H-7′), 7.325 (1H, br s, NH), 7.439 (1H, d, J = 15 Hz, CH=*CH*), and 9.758 (1H, br s, H-1′). 3,4-Dihydroxy-*N*-[2-(5-hydroxyindol-3-yl)ethyl]benzamide (**9**) was synthesized in the same way as described above using serotonin hydrochloride and 3,4-diacetoxybenzoyl chloride. The final product (**9**) was crystallized from ethyl acetate/benzene as fine needles, 18% yield, mp 194–196 °C, FAB-MS m/z 313 ([M+H]$^+$) (calcd $C_{17}H_{16}N_2O_4$ = 312), ^{1}H NMR (270 MHz, acetone-d_6) δ: 2.952 (2H, t, J = 8 Hz, CH_2), 3.621 (2H, m, NCH_2), 6.700 (1H, dd, J = 9 and 2 Hz, H-6′), 6.831 (1H, d, J = 8 Hz, H-5), 7.055 (1H, d, J = 2 Hz, H-4′), 7.101 (1H, br s, H-2′), 7.189 (1H, dd, J = 9 and 1 Hz, H-7′), 7.287 (1H, dd, J = 8 and 2 Hz, H-6), 7.447 (1H, d, J = 2 Hz, H-2), 7.579 (1H, br s, NH), and 9.723 (1H, br s, H-1′). Other compounds (**1**, mp 211–212 °C; **2**, mp 186–187 °C; **4**, mp 211–213 °C; **6**, mp 104–111 °C; **7**, mp 218–221 °C; **8**, mp 151–152 °C; **10**, mp 118–123 °C and 227–231 °C; **11**, mp 212–214 °C; **12**, mp 200–201 °C; **13**, mp 134–168 °C; **14**, mp 141–142 °C (lit.[20] mp 141–142 °C); **15**, mp 200–201 °C; and **19**, mp 212–214 °C (lit.[26] mp 212 °C)) were similarly prepared and identified by elemental analysis, HR-MS, or FAB-MS and/or ^{1}H NMR. **5** was prepared in our previous work.[17] Compounds **16** and **18** were purchased from Tokyo Chemical Industry (Tokyo, Japan) and **17** (hydrochloride) was from Wako Pure Chemical Industries (Osaka, Japan).
11. B16 melanoma cells were grown in DMEM medium containing 15% FBS. Tyrosinase was extracted from the cells by the published method.[5] Assay solutions were prepared by mixing 0.94 ml 50 mM phosphate buffer (pH 6.8) containing 2.6 mM L-DOPA and 10 μl ethanol containing 0–30 mM test compound unless otherwise stated. The reaction was started by adding the enzyme solution (50 μl, containing 2.5–3 mU tyrosinase activity[27]) to the substrate solutions prewarmed to 37 °C, and the dopachrome formation at 37 °C for 5 min was determined by measuring absorbance at 475 nm (ε = 3700 M^{-1} cm^{-1}).[27] Experiments with tyrosinase extracted from HMV-II cells[13] (Dai-Nippon-Sumitomo Pharmaceuticals Inc., Osaka, Japan) and mushroom tyrosinase (Sigma) were done in the same way as described above.
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14. B16 melanoma cells were cultured in 96-well plates (1.5×10^4 cells in 0.2 ml medium per well) for 1 day, and 2 μl ethanol solution containing 0–10 mM test compound was added to each well. The plates were incubated for 2 days and the absorbance of the medium at 415 nm was measured with a plate reader. The medium was aspirated off, and cell viability was assayed with a tetrazolium reagent (CellTiter 96®, Promega). Intracellular melanin content was measured by the published method,[6] but with some modifications. Cells were cultured in 24-well plates (7.5×10^4 cells in 1 ml medium per well) for 1 day, and ethanol solutions (2–6 μl) of test compounds were added to the wells. After 2 days incubation, cells of each well were collected by a scraper, washed with a phosphate-buffered saline, and then homogenized in 0.2 ml 1 N NaOH by sonication. Absorbance at 415 nm of the homogenates was measured to evaluate the intracellular melanin content.
15. Phenolic compounds are often inhibitors as well as substrates for tyrosinase. However, the mixtures of 100 μM **9**, 100 μM **16**, or 100 or 2500 μM **19** with B16 tyrosinase in the assay condition omitting L-DOPA showed no change in the UV–vis spectra (230–610 nm) during an incubation for 60 min, suggesting that oxidation of these compounds by the tyrosinase is, if occurring, negligible in this kinetic study. The spectral data [λ_{max} nm (ε mM^{-1} cm^{-1})] in the phosphate buffer (pH 6.8) were as follows: 258 (13.2) and 286 (11.2) for **9**, 278 (5.7) and 294 (4.9) for **16**, and 256 (9.2) and 290 (5.4) for **19**.
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