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# Methyl *p*-coumarate, a melanin formation inhibitor in B16 mouse melanoma cells

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In honor of Professor Tadao Kamikawa's 70th birthday

**Abstract**—*p*-Coumaric acid (4-hydroxycinnamic acid) and methyl *p*-coumarate (methyl 4-hydroxycinnamate) inhibit the oxidation of L-tyrosine catalyzed by mushroom tyrosinase. However, both were oxidized as monophenol substrate analogues at an extremely slow rate. This oxidation was significantly accelerated as soon as catalytic amounts (0.01 mM) of L-3,4-dihydroxyphenylalanine (L-DOPA) became available as a co-factor. Methyl *p*-coumarate significantly suppressed the melanin formation in B16 mouse melanoma cells, whereas *p*-coumaric acid did not show this activity.


## 1. Introduction

Arbutin, hydroquinone-*O*-β-D-glucopyranoside (**1**), has been widely used as a depigmenting agent in cosmetics (Fig. 1).[1] In our previous paper, arbutin was reported to inhibit the oxidation of L-tyrosine catalyzed by mushroom tyrosinase (monophenolase activity). However, arbutin itself was oxidized as a monophenol substrate at an extremely slow rate. This oxidation was accelerated as soon as catalytic amounts (0.01 mM) of L-3,4-dihydroxyphenylalanine (L-DOPA) became available as a co-factor.[2] In addition, the recent report indicates that *o*-phenylphenol, which is known as a depigmenting agent, has little depigmenting action while its metabolite, phenylhydroquinone is a potent depigmenting agent affecting melanocytes.[3] Hence, it may not be illogical to assume that depigmenting action involves quinone formation catalyzed by tyrosinase. This prompted us to search for monophenol substrate analogues, which may possess depigmenting activity. In previous papers, the isolation of *p*-coumaric acid (**2**) from the leaves of *Panax ginseng* (Araliaceae) and methyl *p*-coumarate (**3**) from the flower of *Trixis michuacana* var *longifolia* (Compositae) were reported[4] and hence,

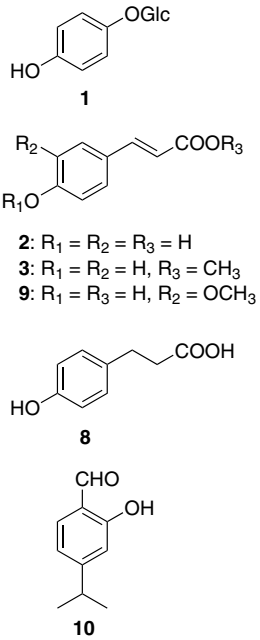


**Figure 1.** Chemical structures of *p*-coumaric acid and its related phenylpropanoids.

*Keywords*: Mushroom tyrosinase; Monophenolase; *p*-Coumaric acid; Methyl *p*-coumarate; B16 mouse melanoma cell.
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these phenylpropanoids were re-examined if both exhibit depigmenting activity.

## 2. Results and discussion

Tyrosinase (EC 1.14.18.1) contains a strongly coupled binuclear copper active site and functions both as a monophenolase and as an *o*-diphenolase.[5,6] The enzymatic reaction can be detected by spectrophotometrical (dopachrome formation) and polarographical (oxygen consumption) methods. Since *p*-coumaric acid is classified as a monophenol analogue like arbutin, which has a possibility to be oxidized by *oxy*-tyrosinase, the incubation experiments were performed by measuring oxygen consumption up to 20 min (Fig. 2, line 1). Although *p*-coumaric acid consumed a small amount of oxygen (20 μM), its UV–vis absorption spectrum did not noticeable change up to 20 min (data not illustrated). In addition, HPLC analysis confirmed that *p*-coumaric acid remained without any change up to 20 min as shown in Figure 3. However, oxygen consumption was significantly accelerated as soon as a catalytic amount (0.01 mM) of L-DOPA became available (Fig. 2, line 4). HPLC analysis indicates that *p*-coumaric acid started being oxidized, presumably to caffeic acid (**4**) at an extremely slow rate. This was consistent with the result from co-injection with an authentic sample of caffeic acid.[7] As soon as a co-factor became available, HPLC analysis demonstrated that *p*-coumaric acid decreased and the peak '***b***' corresponding to caffeic acid increased, and the changes in its UV–vis absorption spectrum started in increasing absorbance at 260 and 360 nm, characteristic of *o*-quinone (Figs. 4 and 5). It appears that *p*-coumaric acid was oxidized as a substrate to caffeic acid, followed further oxidation to the corresponding *o*-quinone (**5**) (Scheme 1).[7,8] Similar to arbutin,[2] *p*-coumaric acid seems to possess tyrosinase inhibitory activity by following the enzymatic reaction at dopachrome formation. However, an observation by spectrophotometer led to an unavoidable consideration when these compounds could act as monophenol

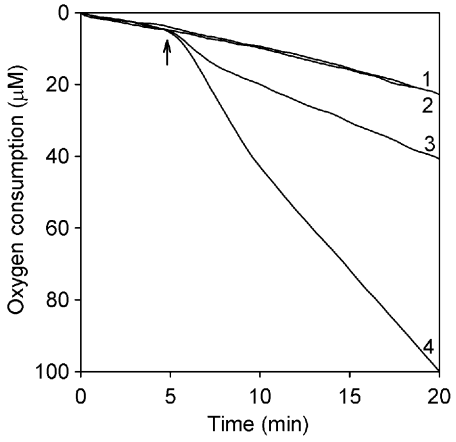



**Figure 2.** Oxygen consumption of *p*-coumaric acid (1,4) and methyl *p*-coumarate (2,3) in presence of tyrosinase. The concentration was selected at 0.5 mM. The arrow (↑) indicated the time when catalytic amount (10 μM) of L-DOPA was added (3,4).

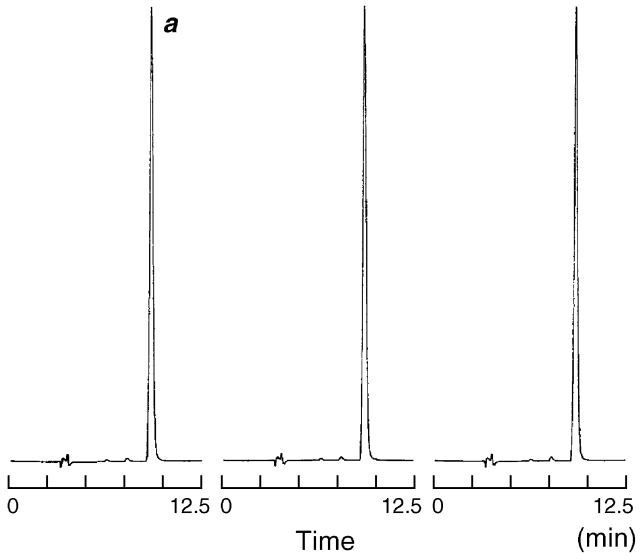



**Figure 3.** HPLC analysis of the reaction medium with 0.5 mM of *p*-coumaric acid and tyrosinase. Sampling time was chosen at 0 min (left), 10 min (middle), and 20 min (right), respectively. The HPLC operating conditions was described in Section 3 in details.

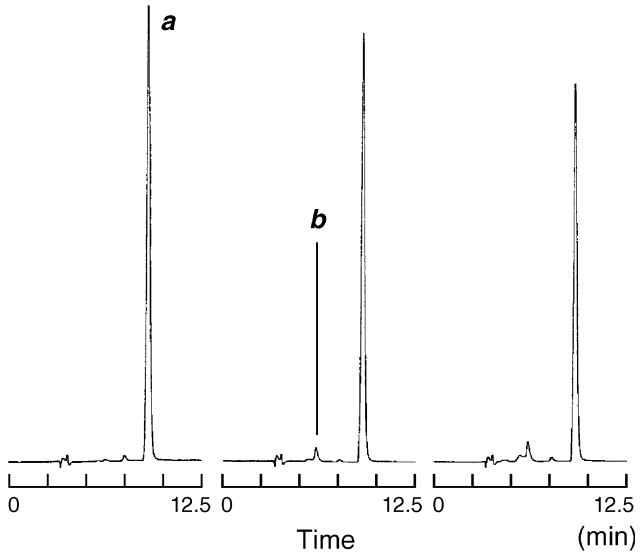



**Figure 4.** HPLC analysis of the reaction medium with 0.5 mM of *p*-coumaric acid and tyrosinase. The catalytic amount of L-DOPA (10 μM) was added at 5 min. Sampling time was chosen at 0 min (left), 10 min (middle), and 20 min (right), respectively. The HPLC operating conditions was described in Section 3 in details.

substrates and the corresponding products showed insignificant absorption at 475 nm. The result suggests that *p*-coumaric acid eventually inhibited dopachrome formation from L-tyrosine catalyzed by mushroom tyrosinase if a co-factor is not available, but it does not inhibit the oxidation of L-DOPA.

Subsequently, oxygen consumption of methyl *p*-coumarate (**3**) was also measured for comparison. The results obtained are similar to those observed for *p*-coumaric acid. Methyl *p*-coumarate did not significantly consume

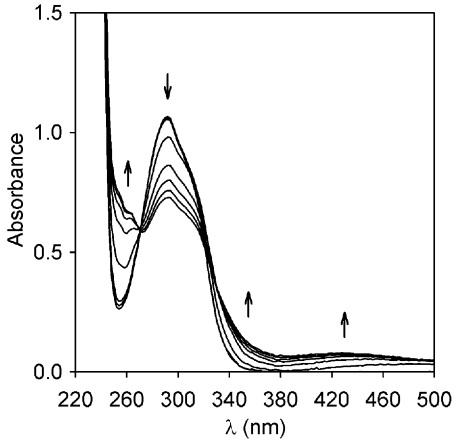
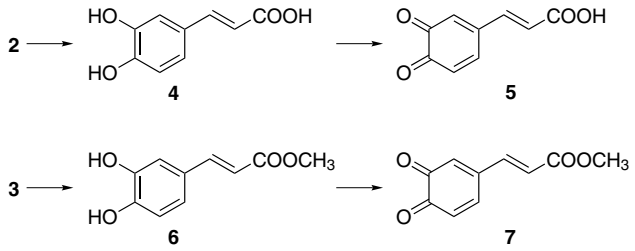



**Figure 5.** Consecutive spectra obtained in the oxidation of 0.5 mM of *p*-coumaric acid by tyrosinase for 20 min. The catalytic amount of L-DOPA (10 μM) was added at 5 min. Scan speed was at 2 min intervals for 30 s. The arrows (↑) designate the evolution of the peak.




**Scheme 1.** Oxidation of *p*-coumaric acid or methyl *p*-coumarate to corresponding compounds by tyrosinase.

oxygen up to 20 min as shown in Figure 2. The UV–vis absorption spectrum did not change up to 20 min (data not illustrated). However, as soon as a catalytic amount (0.01 mM) of L-DOPA became available, oxygen consumption was accelerated but this rate was slower than that by *p*-coumaric acid. Moreover, addition of L-DOPA induced UV–vis spectrum change (Fig. 6). Presumably, methyl *p*-coumarate is oxidized to the corresponding *o*-diol (**6**) and then to *o*-quinone (**7**) when a co-factor is available (Scheme 1). The resulting quinone is unstable molecules susceptible of undergoing a variety of self-driven reactions. The result is consistent with the previous report that methyl *p*-coumarate did not inhibit the oxidation of L-DOPA catalyzed by mushroom tyrosinase.[4] However, the slow enzymatic oxidation of methyl *p*-coumarate possibly indicates that this compound coordinates to one copper like a monophenol substrate analogue and positions over the binuclear active site competitively.

Oxygen consumption of dihydrocoumaric acid (**8**) and ferulic acid (**9**) were also monitored in the presence of tyrosinase for comparison. In the case of dihydrocoumaric acid, it consumed oxygen immediately even without a co-factor (Fig. 7, inset). In the UV–vis absorption spectrum, the changes started with increasing absorbance at 260, 280, 330, 400, and 470 nm as shown in Figure 7. Evidently, dihydrocoumaric acid was easily oxidized as a substrate. Its carboxylate is no longer conjugated into the aromatic ring indicating that this conjugation is important to elicit the inhibitory property as a substrate analogue toward *met*-tyrosinase. The structure of **8** resembles *p*-cresol, which has been frequently employed as an experimental substrate.[9,10] In contrast, ferulic acid hardly consumed oxygen and its UV–vis absorption spectrum was inconsequential change (data not illustrated). The results obtained indicate that this phenolic acid may not be easy to bind with the active site because of steric hindrance by an adjacent methoxy group.

The discussion previously reported[4] was mainly on the basis of the experiment using L-DOPA as a substrate. Therefore, the activity described is *o*-diphenolase inhibitory activity of mushroom tyrosinase. It should be noted that the lag time is known for the oxidation of monophenolic substrates such as L-tyrosine to L-DOPA. This lag can be shortened or abolished by the presence

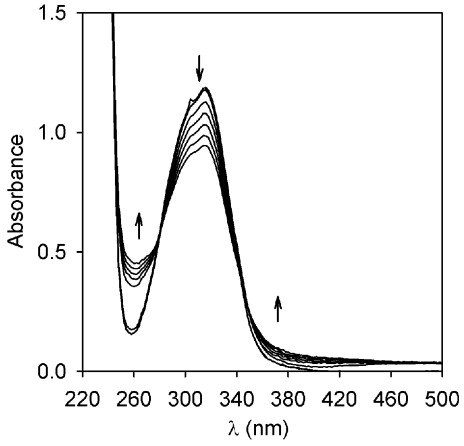



**Figure 6.** Consecutive spectra obtained in the oxidation of 0.5 mM of methyl *p*-coumarate by tyrosinase for 20 min. The catalytic amount of L-DOPA (10 μM) was added at 5 min. Scan speed was at 2 min intervals for 30 s. The arrows (↑) designate the evolution of the peak.

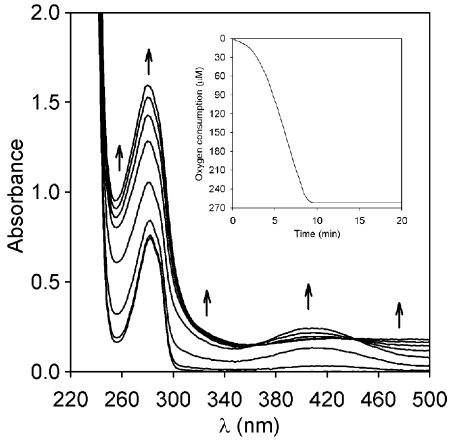



**Figure 7.** Consecutive spectra obtained in the oxidation of 0.5 mM of dihydrocoumaric acid by tyrosinase for 20 min. The arrows (↑) designate the evolution of the peak. The inset shows oxygen consumption of dihydrocoumaric acid (0.5 mM) by tyrosinase.

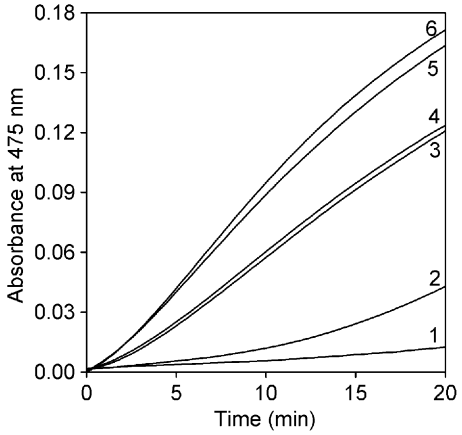



**Figure 8.** Inhibitory effect on the rate of dopachrome formation from L-tyrosine (0.125 mM) catalyzed by tyrosinase. The concentration of methyl *p*-coumarate (1), *p*-coumaric acid (2), 4-methoxycinnamic acid (3), cinnamic acid (4), and ferulic acid (5) were 0.25 mM. Curve 6 represents the oxygen consumption without inhibitor.

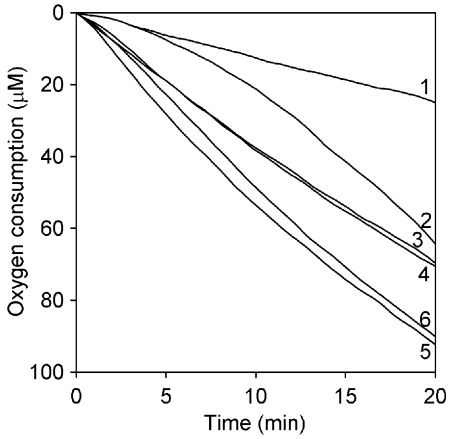


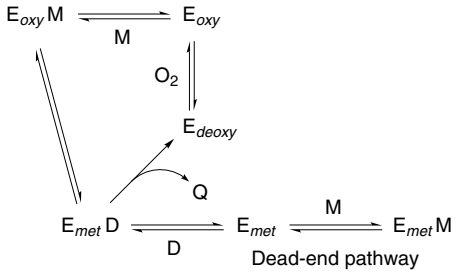

**Figure 9.** Inhibitory effect on the rate of oxygen uptake by L-tyrosine (0.125 mM) catalyzed by tyrosinase. The concentration of methyl *p*-coumarate (1), *p*-coumaric acid (2), 4-methoxycinnamic acid (3), cinnamic acid (4), and ferulic acid (5) were 0.25 mM. Curve 6 represents the oxygen consumption without inhibitor.

of reducing agents (co-factors), especially *o*-diphenols (such as L-DOPA and caffeic acid). On the other hand, inhibitors for monophenolase activity are known to extend the lag phase. Thus, inhibitory effects on the enzymatic oxidation of L-tyrosine by several coumaric acid derivatives and their benzoic acid counterparts were compared by spectrophotometric and polarographic methods. Interestingly, *p*-coumaric acid and methyl *p*-coumarate significantly lengthened this lag phase, indicating that both act as effective inhibitor with regard to dopachrome formation. Those inhibitory effects can be placed in decreasing order methyl *p*-coumarate > *p*-coumaric acid > 4-methoxycinnamic acid = cinnamic acid, at the result as shown in Figure 8. In connection with cinnamic acid, its methyl ester was noted to be an inhibitor with an $IC_{50}$ of 2.4 mM without being oxidized, indicating that the general presence of phenolic hydroxyl group is not a prerequisite for the inhibition. Although this activity is weak, the result may provide a hint to search for tyrosinase inhibitors without being oxidized.

However, at polarographic observation, *p*-coumaric acid showed gradually slow acceleration after the reaction was started (Fig. 9). These differences between spectroscopic and polarographic experiments were possibly originated by facile oxidation property of *p*-coumaric acid, and can be considered from the conversion of *met*-tyrosinase to *oxy*-tyrosinase by a diphenol that its product emerged in the assay system.[11] However, methyl *p*-coumarate inhibited effectively the enzymatic oxidation of L-tyrosine, which was confirmed spectrophotomerically as well as polarographically, although this methyl ester is slightly oxidized by generated *oxy*-tyrosinase. Notably, methyl coumarate was found to be a most potent monophenolase inhibitor in the coumaric acids tested. Hence, esterification of *p*-coumaric acid resulted in increasing activity. In the case of diphenolase inhibitory activity, esterification of the carboxylic group decreased the inhibitory strength,[12] and more importantly, the ester is no longer an inhibitor but a substrate.

The inhibition activity exerted by *p*-coumaric acid and methyl *p*-coumarate could be on the basis of the assumption that both bind as a monophenol substrate analogue (M) to the *met*-form of tyrosinase ($E_{met}$), and the resulting $E_{met}M$ complex is inactive. This postulate is consistent with the fact that monophenols can only react with the *oxy*-form of tyrosinase in an ordered sequential mechanism.[13] Thus, the $E_{met}M$ is the dead-end complex.[5] However, the commercial tyrosinase is known to contain a small amount of the *oxy*-form ($E_{oxy}$). If this is so, the $E_{oxy}$ would start the turnover by acting on M, which is hydroxylated to generate $E_{met}D$. At this point the enzyme may oxidize *o*-diphenol (D) to the corresponding *o*-quinone (Q) generating $E_{deoxy}$, or release D producing $E_{met}$ as illustrated in Scheme 2. In the case of *p*-coumaric acid, D is caffeic acid, which is known to act as a co-factor. If D is released, it can be recycled to the reaction medium through intramolecular cyclation and further redox steps. Similar to arbutin,[2] both *p*-coumaric acid and its methylester were oxidized as monophenol substrates to the corresponding *o*-diols at an extremely slow rate. Once oxidized to *o*-diols, they were further oxidized to the corresponding *o*-quinone rather quickly.

Subsequently, the effects of *p*-coumaric acid and methyl *p*-coumarate on melanin formation in B16 mouse melanoma cells were examined. Arbutin was also tested




**Scheme 2.** Action mechanism of tyrosinase on monophenol substrates.

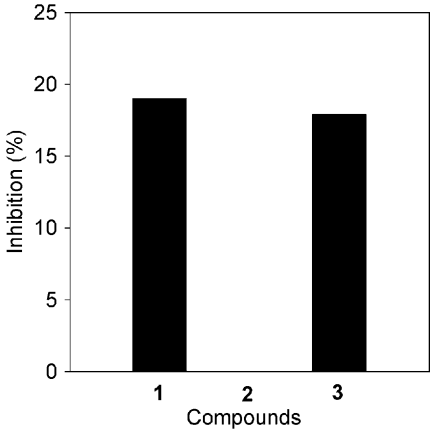



**Figure 10.** Inhibitory effect on melanin formation using B16 mouse melanoma cell. Inhibition (%) was estimated by comparison of absorbance at 475 nm with drug-free experiment. The concentrations of arbutin (**1**) and *p*-coumaric acid (**2**) were 0.1 mM, respectively. The concentration of methyl *p*-coumarate (**3**) was 0.01 mM.

for comparison. The melanin production was significantly suppressed when the cells were cultured with 10 μM of methyl *p*-coumarate (Fig. 10). This cellular depigmenting activity was about 10-fold more potent than that of arbutin. The precise explanation for the depigmenting activity remains to be clarified but it likely acts similar to arbutin.[14] Notably, *p*-coumaric acid did not suppress the melanin formation in cultured melanocytes at 100 μM. This difference likely comes from the fact that deprotonated *p*-coumaric acid may not enter the cells. The cellular depigmenting activity of methyl *p*-coumarate was observed without inhibiting cell growth. The possibility that the depigmenting activity observed might be caused in part by its cytotoxicity[15] may not be entirely ruled out. However, the cytotoxic activity of methyl *p*-coumarate against B16 mouse melanoma cells and several solid tumor cell lines was observed at 500 μM. Hence, the depigmenting activity of the cells by methyl coumarate may be expected at dose levels causing no cytotoxicity.

It is not clear if the inhibition of the melanin formation is caused either methyl *p*-coumarate inhibits tyrosinase or the oxidized metabolites is involved in the melanocyte. The latter case is more like for methyl *p*-coumarate because chamaecin (2-hydroxy-4-isopropylbenzaldehyde) (**10**) has recently been reported as a potent tyrosinase inhibitor[16] but it did not suppress the melanin formation in B16 mouse melanoma cells at all at the concentration of 100 μM. It should be noted, however, that the relevance of the results of in vitro experiments in simplified systems to the cellular depigmenting activity need to be carefully considered. The further study is currently under investigation.

On the basis of an Hückel theory calculation for tyrosinase active site model, it has been shown that ionization of hydroxyl group of phenolic compound is a crucial step in its interaction with positively charged copper of the active site in monophenolase reaction.[17] Accordingly, it is assumed that an electron-withdrawing group increased the phenol's affinity toward an active site by increasing its acidity. This assumption can be applicable to explain the inhibitory behaviors by *p*-coumaric acid and methyl *p*-coumarate since both possess the conjugated carboxyl group. On the other hand, direct interaction with *oxy*-tyrosinase has been observed spectroscopically in the absence of the phenolic substrate, and coordination of the carboxylate group to one copper of the binuclear site was suggested.[13] Methyl *p*-coumarate possesses an affinity toward active site by its phenolic hydroxyl group whereas *p*-coumaric acid binds to copper site for being two moieties, carboxylate and phenolic hydroxyl. The latter bidentate coordination is possibly attributable to the inhibitory activity of *p*-coumaric acid less than that of methyl *p*-coumarate. Since dihydrocoumaric acid is oxidized easily by tyrosinase, a valance of the electron density and structural feature by phenolic hydroxyl and styrene is important in order to conserve high affinity on active site with preventing its easy dissociation and oxidation.

The results obtained so far may indicate a hint to their interaction with the enzyme but this remains unclear since the structure of mushroom tyrosinase used for this study has not yet been established. However, safety is a primary consideration for tyrosinase inhibitors, especially for those in cosmetic products, which may be utilized in unregulated quantities on a regular basis. Compared to other depigmenting agents, *p*-coumaric acid and its methyl ester are almost colorless and odorless, and therefore are superior cosmetic additive, particularly to inhibit the oxidation of L-tyrosine. Interestingly, one molecule of *p*-coumaric acid scavenges one molecule of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical at an extremely slow rate, while methyl *p*-coumarate does not have this scavenging activity. In addition, substituted derivatives of cinnamic acid are predominant phenolic acids present in many edible plants.[18] The phenylpropanoids play important roles in UV protectants.[19]

## 3. Experimental

### 3.1. Materials

Arbutin was available from our previous work[20] and also purchased from Sigma Chemical Co. (St. Louis, MO). Methyl *p*-coumarate was from our previous work.[4] Caffeic acid, *p*-coumaric acid, dimethylsulfoxide (DMSO), 4-methoxycinnamic acid, and trifluoroacetic acid (TFA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Cinnamic acid, dihydrocoumaric acid, ferulic acid, and mushroom tyrosinase were purchased from Sigma Chemical Co. Acetonitrile (MeCN) for HPLC was purchased from Alfa Aesar Chemical Co. (Ward Hill, MA). 4-*tert*-Butylcatechol was purchased from Fluka Chemical Co. (Milwaukee, WI). Mushroom tyrosinase was purified by the procedure as previously reported.[21]

### 3.2. Enzyme assay

The assay was performed as previously reported with some modifications.[22] Oxygen consumption was

measured by Clark style electrode and an oxygraph equipped (YSI-53) with a water-jacketed chamber (Yellow Springs Instrument Co., Yellow Springs, OH) maintained at 30 °C. Calibration of an oxygen electrode was performed by using 4-*tert*-butylcatechol and excess tyrosinase according to the previous report.[23] Absorption measurements were recorded by a Spectra MAX plus spectrophotometer (Molecular Device, Sunnyvale, CA) at 30 °C. All samples were first dissolved in DMSO at 150 mM and used for the experiment with dilution. The final concentration of DMSO in the test solution is always 3.3%. For obtaining data of absorbance increment at 475 nm and oxygen consumption, 0.1 mL of a inhibitor solution was mixed with 0.6 mL of 0.25 M sodium phosphate buffer (pH 6.8) and 2.2 mL of water. Then, 0.1 mL of the 0.05 M sodium phosphate buffer solution (pH 6.8) of tyrosinase (1.0 μg/mL) was added. When L-tyrosine was used as monophenol substrate, instead of 2.2 mL of water, 0.3 mL of 1.25 mM L-tyrosine solution and 1.9 mL of water were added. In the experiment by using co-factor, 6 μL of DOPA (5 mM) was added at 5 min after enzymatic reaction started. Scan speed was selected for 2 min with 30 s intervals for obtaining consecutive spectra (220–500 nm).

### 3.3. HPLC analysis

HPLC analysis was performed as previously reported with slight modification.[2] The analysis was performed on an EYELA LPG-1000 with an EYELA UV-7000 detector (EYELA, Tokyo) and Capcell Pak C-18 column (5 μm, 4.6 × 250 mm, Shiseido, Tokyo). In general, the operating conditions were as follows: solvent; 20% MeCN/$H_2O$ containing 0.1% TFA, flow rate; 0.8 mL/mim, detection; UV at 280 nm, injected amount; 10 μL from 3 mL assay system. The retention time ($t_R$) of *p*-coumaric acid or caffeic acid were identified as 9.3 or 6.3 min, respectively. When 15% MeCN/$H_2O$ containing 0.1% TFA used as HPLC solvent, *p*-coumaric acid or caffeic acid showed $t_R$ 15.9 or 8.3 min, respectively. Both peaks were identified by the co-injection of standard compounds.

### 3.4. Cell culture and melanin assay

The cell culture and melanin assay were performed as previously reported with slight modification.[14] Briefly, B16 mouse melanoma cells were donated from Institute of Development, Aging and Cancer, Tohoku University, Japan, were cultured in RPMI1640 medium in a humidified atmosphere with 10% $CO_2$ at 37 °C. Subcultures of cells were plated at a density of $2.0 \times 10^5$ cells/well. Approximately 24 h later, the sample was added, and cells were harvested for 72 h after drug addition. All samples were dissolved in DMSO and the final DMSO concentration was 0.1% in all experiments. The harvested cells were suspended in 0.5 mL of 1 M NaOH solution containing 10% DMSO, which were sonicated and incubated overnight. The inhibition of melanin formation was estimated by the comparison of absorbance at 475 nm with inhibitor-free experiment. The cytotoxicity assay was performed as previously described.[24]

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