2,4-Dihydroxycinnamic Esters as Skin Depigmenting Agents

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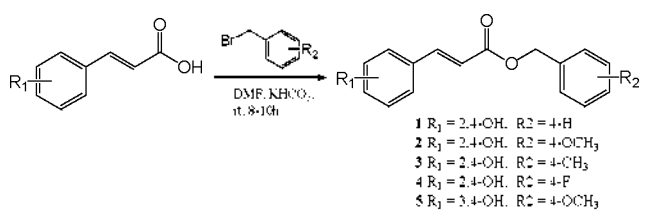
Melanogenesis refers to the biosynthesis of melanin pigments in melanocytes and is controlled by both the intra- and extracellular environment.[1] Melanogenesis is initiated by tyrosinase, which oxidizes tyrosine to dopaquinone; dopaquinone is subsequently converted to pheomelanin, a pale yellow/red compound, and eumelanin, which is light brown to black in color.[2-4] In the skin, melanin affects pigmentation and protects the skin from ultraviolet radiation and other causes of dermal damage.[5] while abnormal accumulation of melanin can lead to melasma and freckles.[6] Therefore, tyrosinase inhibitors may be employed as depigmenting agents to treat melanin hyperpigmentation.[7] Although numerous depigmenting agents composed of natural and synthetic compounds have been reported, widespread application in cosmetics and pharmaceuticals has been limited due to severe side effects and cytotoxicity. Current commercial depigmenting agents include hinokitiol, linoleic acid, and kojic acid.[8]

Recent research on synthetic depigmenting agents has explored the use of alkoxy benzoate and alkoxy cinnamate derivatives,[9] and hydroxamic acids.[10] Other studies have focused on synthesizing derivatives of known tyrosinase inhibitors such as 5-[(3-aminopropyl) phosphinooxy]-2-(hydroxymethyl)-4*H*-pyran-4-one.[11] and *N*-hydroxycinnamoylphenalkylamide derivatives.[12] Both the biological effects and safety regulations must be considered in the development of skin depigmenting agents since these compounds can induce undesirable biochemical reactions.[9] Many skin depigmenting agents exhibit inhibitory effects on melanogenesis through biological activities including tyrosinase inhibition.[13,14] In this study, we synthesized 2,4-dihydroxycinnamic esters and tested cytotoxicity with MTT assay and inhibitory activities using DPPH assay, melanin content assay, and mushroom tyrosinase assay.

Results and Discussion

The general synthetic pathway for dihydroxycinnamic ester derivatives is shown in Scheme 1. The reaction of 2,4-dihydroxycinnamic acid with a phenyl-substituted alkyl bromide was conducted in the presence of $KHCO_3$ at room temperature, and provided reasonable yields with benzyl bromide. The reaction products consisted of multiple compounds differing by the substituents on the benzyl ester component. 3,4-Dihydroxycinnamic ester (**5**) was also synthesized as a reference compound for activity comparisons.



Scheme 1

The cytotoxicity of compounds was determined with an MTT assay. The degree of inhibitory activity on melanogenesis and melanin formation was determined using a melanin content assay. The results are presented in Table 1.

Regardless of their suitability for pharmaceuticals and cosmetics, all compounds were tested for inhibition of melanin formation (IC_{50}). Low cytotoxicity (IC_{50} > 100 μM) was observed for 2,4-dihydroxycinnamic ester derivatives (**1-4**), and the 3,4-dihydroxycinnamic ester (**5**) exhibited a moderate cytotoxicity (IC_{50} = 53 μM). To determine biological activity, the effect of 2,4-dihydroxycinnamic esters (**1-4**) on melanin biosynthesis in B16/F1 murine melanoma cells was investigated. IC_{50} values for kojic and caffeic acids in B16/F1 murine melanoma cells were 366.8 μM and 230.9 μM, respectively. All 2,4-dihydroxycinnamic esters were effective at inhibiting melanin production with a substituent effect on melanogenesis inhibition increasing as -F > -CH_3 > -H > -OCH_3. The 4-fluoro-substituted benzyl ester (**4**) exhibited superior inhibition activity relative to that of both kojic and caffeic acids and was slightly more effective than the other 2,4-dihydroxycinnamic esters (**1-3**).

Since tyrosinase catalyzes an oxidation reaction, antioxidants may inhibit melanogenesis without directly interacting with tyrosinase.[11] To differentiate between direct inhibition

Table 1. Inhibition of melanin formation and cytotoxicity by dihydroxycinnamic ester derivatives

Compound	Inhibition of melanin formation IC_{50} (μM)	Cytotoxicity IC_{50} (μM)
1	40.8	> 100
2	45.1	> 100
3	37.0	> 100
4	34.7	> 100
5	4.3	53
Caffeic acid[a]	230.9	> 100
Kojic acid[a]	366.8	> 100

[a]Reference compounds.

Table 2. Tyrosinase Inhibitory activity and antioxidant activity of dihydroxycinnamic ester derivatives

Compound	Tyrosinase inhibitory activity IC_{50} (μM)	Antioxidant activity[a] IC_{50} (μM)
1	0.9	> 300
2	0.8	> 300
3	0.7	> 300
4	0.5	> 300
5	> 300	24.3
2,4-Dihydroxy-cinnamic acid	7.8	> 300
Caffeic acid[b]	> 300	24.5
Kojic acid[b]	211.9	> 300
Ascorbic acid[b]	> 300	26.7

[a]DPPH radical scavenging activity. [b]Reference compounds.

and antioxidant activity of 2,4-dihydroxycinnamic esters (**1-4**), tyrosinase inhibitory activity was measured with a mushroom tyrosinase assay and the antioxidant activity was measured with a DPPH assay. The results are shown in Table 2.

Compounds **1-4** showed a high degree of tyrosinase inhibitory activity, but exhibited low antioxidant activity. The biological activity results indicated that the observed depigmentation effects of compounds **1-4** were the results of tyrosinase inhibition rather than antioxidant activity. However, useful skin depigmenting agents must be both effective and safe for application in pharmaceuticals and cosmetics. The results indicate that 2,4-dihydroxycinnamic ester derivatives (**1-4**) significantly reduced tyrosinase activity and melanin synthesis with low cytotoxicity.

Conclusion

The synthetic 2,4-dihydroxycinnamic esters (**1-4**) induced a reduction in the melanogenesis with relatively low cytotoxicity. The results of the comparative mushroom tyrosinase and antioxidant assays indicated that the observed depigmentation effects of **1-4** were the results of tyrosinase inhibition. Although the synthesized compounds cannot be directly used in commercial applications such as cosmetics and pharmaceuticals, the excellent biological activity of 2,4-dihydroxycinnamic esters indicates the potential of this class of compounds for application in skin depigmentation treatments.

Experimental Section

Instruments. Melting points were determined using a Thermo Scientific Electro thermal 9100 melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-AL400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Mass spectra were obtained using a Waters Quattro premier XE mass spectrometer and UV spectra using a Molecular Devices Spectra Max microplate reader.

Materials. 5-Hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one (Kojic acid), caffeic acid, 2,4-dihydroxycinnamic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and ascorbic acid were purchased from Aldrich Chemical Co. All other chemicals and solvents were used with commercially available highest grade.

General procedure for the synthesis of compound 1.[15] 2 mM 2,4-Dihydroxy cinnamic acid was dissolved in 10.0 mL DMF, and 2.4 mM $KHCO_3$ was added slowly. The resulting mixture was stirred for several minutes at room temperature. Then 3.0 mM benzyl bromide was added and stirred for 8-10 hours at room temperature. Upon completion, the reaction mixture was added to water and extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous magnesium sulfate. The solution was filtered and concentrated. The residue was purified with column chromatography using ethyl acetate-hexane = 3:1 as an eluent. The following compounds were prepared using the above mentioned general procedure.

Benzyl 3-(2,4-dihydroxyphenyl)acrylate (1). Yield 58%; white solid; mp 142-144 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 8.00 (d, 1H, J = 16.0 Hz), 7.48 (d, 1H, J = 8.4 Hz), 7.45-7.29 (m, 5H), 6.53 (d, 1H, J = 16.0 Hz), 6.51 (d, 1H, J = 2.4 Hz), 6.45 (dd, 1H, J = 2.4 Hz, J = 8.4 Hz), 5.23 (s, 2H); ^{13}C NMR (100 MHz, acetone-d_6) δ 167.9, 161.6, 159.1, 141.5, 137.8, 131.3, 129.2, 128.8, 128.7, 114.7, 114.5, 108.9, 103.5, 66.1; MS *m/z* 269 [M-H]$^-$.

4-Methoxybenzyl 3-(2,4-dihydroxyphenyl)acrylate (2). Yield 40%; white solid; mp 143-145 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.97 (d, 1H, J = 16.0 Hz), 7.46 (d, 1H, J = 8.4 Hz), 7.38 (d, 2H, J = 8.4 Hz), 6.93 (d, 2H, J = 8.4 Hz), 6.49 (d, 1H, J = 2.4 Hz), 6.48 (d, 1H, J = 16.0 Hz), 6.44 (dd, 1H, J = 2.4 Hz, J = 8.4 Hz), 5.14 (s, 2H), 3.79 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ 168.0, 161.6, 160.5, 159.1, 141.3, 131.3, 130.8, 129.7, 114.9, 114.5, 108.9, 103.5, 65.9, 55.4; MS *m/z* 299 [M-H]$^-$.

4-Methylbenzyl 3-(2,4-dihydroxyphenyl)acrylate (3). Yield 51%; white solid; mp 150-152 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.98 (d, 1H, J = 16.0 Hz), 7.46 (d, 1H, J = 8.4 Hz), 7.32 (d, 2H, J = 8.4 Hz), 7.18 (d, 2H, J = 8.4 Hz), 6.50 (d, 1H, J = 16.0 Hz), 6.49 (d, 1H, J = 2.4 Hz), 6.44 (dd, 1H, J = 2.4 Hz, J = 8.4 Hz), 5.17 (s, 2H), 2.31 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ 167.9, 161.7, 159.1, 141.4, 138.3, 134.8, 131.4, 129.8, 129.0, 114.8, 114.5, 108.9, 103.5, 66.0, 21.1; MS *m/z* 283 [M-H]$^-$.

4-Fluorobenzyl 3-(2,4-dihydroxyphenyl)acrylate (4). Yield 44%; white solid; mp 173-175 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.99 (d, 1H, J = 16.0 Hz), 7.51-7.46 (m, 3H), 7.17-7.12 (m, 2H), 6.51 (d, 1H, J = 16.0 Hz), 6.50 (d, 1H, J = 2.4 Hz), 6.44 (dd, 1H, J = 2.4 Hz, J = 8.4 Hz), 5.20 (s, 2H); ^{13}C NMR (100 MHz, acetone-d_6) δ 167.9, 164.4, 162.0, 161.7, 159.1, 141.6, 134.0, 131.4, 131.1, 116.1, 115.8, 114.6, 114.5, 108.9, 103.5, 65.4; MS *m/z* 287 [M-H]$^-$.

4-Methoxybenzyl 3-(3,4-dihydroxyphenyl)acrylate (5). Yield 47%; white solid; mp 166-168 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.57 (d, 1H, J = 16.0 Hz), 7.38 (d, 2H, J = 8.4 Hz), 7.16 (d, 1H, J = 2.4 Hz), 7.05 (dd, 1H, J = 2.4 Hz, J = 8.4 Hz), 6.94 (d, 2H, J = 8.4 Hz), 6.87 (d, 1H, J = 8.4 Hz), 6.31 (d, 1H, J = 16.0 Hz), 5.14 (s, 2H), 3.80 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ 167.3, 160.4, 148.8, 146.3, 145.8, 130.8, 129.6, 127.6, 122.5, 116.3, 115.5, 115.2, 114.6, 66.1, 55.5; MS *m/z* 299 [M-H]$^-$.

Cell culture. B16F1murine melanoma cells were purchased from the American Type Culture Collection. The cells were grown in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA). The cells were maintained in a humidified incubator with 5% CO_2 at 37 °C. All the tested compounds were prepared in DMSO.

MTT assay. The MTT assay described by Mosmann.[16,17] Briefly, the cell suspension was seeded in a microplate and the cells were allowed to completely adhere to the plate overnight. Each test compound was added to the plate, and the plates were incubated at 37 °C in a CO_2 incubator. After 24 h, 10 μL of the MTT solution (5 mg/mL in PBS) was added to each cell, and the incubation was continued for 4 h. The supernatant was then removed and formazan was solubilized by adding 100 μL of DMSO to each well, with gentle shaking. The absorbance was then measured using a microplate reader at 570 nm.

Mushroom tyrosinase assay. The tyrosinase activity was determined using the method described by Masamoto *et al.* with a slight modification.[18-20] Mushroom tyrosinase, 3-(3,4-dihydroxyphenyl)-L-alanine (L-dopa) were purchased from Sigma Chemical Co (St. Louis, Mo, USA). The test compound solution was prepared by mixing the compound with DMSO and a 0.1 M phosphate buffer (pH 7.0). Then a test tube was filled with 80 μL of the 0.1 M phosphate buffer (pH 7.0), 40 μL of the test compound solution, and 40 μL of mushroom tyrosinase (1,500-2,000) U/μL. Then 40 μL of 2.5 mM L-dopa was added. The same control solution without the test compound was also prepared. The UV absorbance was measured using a microplate reader at 475 nm, and the percent inhibitions (%) were calculated using the following formula:

$$\text{Percent inhibitions (\%)} = [(Ac-As)/Ac] \times 100$$

where Ac and As are the absorbance for the control and for the samples, respectively. Each sample was assayed at different concentrations.

DPPH assay. The DPPH assay was based on the reported methods.[21,22] To determine the capacity to scavenge the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH), each test compounds were mixed with 100 μL of DPPH radical in methanol solution (0.2 mM). After a 20 min incubation at room temperature, the absorbance was read at 517 nm using a microplate reader. The DPPH radical scavenging activity was expressed as follow:

$$\text{DPPH radical scavenging activity (\%)} = [(Ac-As)/Ac] \times 100$$

Where Ac was the absorbance of the control, As was the absorbance of the sample. Each sample was assayed at different concentrations.

Melanin content assay. The melanin content was measured using the method reported by Shimizu *et al.* with a slight modification.[23] Briefly, B16F1 murine melanoma cells were rinsed in phosphate-buffer saline (PBS) and removed using 0.05% trypsin / EDTA. The cells were incubated overnight in density 1×10^5 cells in six-well plates, and then treated with the test compound at various concentrations in phenol red-free DMEM for 3 d. After the medium was removed and the cells were washed with PBS, they were dissolved in 1 N NaOH that contained 10% DMSO. Then the relative melanin content was determined based on the optical densities (OD) at 405 nm using a microplate reader.

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Reference

1. Cho, Y. H. *J. Eep. Bioned. Sci.* **2007**, *13*, 313.
2. Choi, S. Y.; Hwang, J. S.; Kim, S. H.; Kim, S. Y. *Biochem. Biophys. Res. Commun.* **2006**, *349*, 39.
3. Shiino, M.; Watanabe, Y.; Umezawa, K. *Bioorg. Chem.* **2003**, *31*, 129.
4. Mason, H. S.; Peterson, E. W. *Biophys. Acta* **1965**, *111*, 134.
5. Hwang, J. S.; Choi, H.; Rho, H. S.; Shin, H. J.; Kim, D. H.; Lee, J.; Lee, B. G.; Chang, I. *Br. J. Dermatol.* **2004**, *150*, 39.
6. Jeon, S. H.; Kim, K. H.; Koh, J. U.; Kong, K. H. *Bull. Korean Chem. Soc.* **2005**, *26*, 1135.
7. Khan, K. M.; Maharvi, G. M.; Khan, M. T. H.; Shaikh, A. J.; Perveen, S.; Begum, S.; Choudhary, M. I. *Bioorg. Med. Chem.* **2006**, *14*, 344.
8. Roh, J. S.; Han, J. Y.; Kim, J. H.; Hwang, J. K. *Biol. Pharm. Bull.* **2004**, *27*, 1976.
9. Kang, H. H.; Rho, H. S.; Hwang, J. S.; Oh, S. K. *Chem. Pharm. Bull.* **2003**, *51*, 1085.
10. Baek, H. S.; Rho, H. S.; Yoo, J. W.; Ahn, S. M.; Lee, J. Y.; Lee, J. A.; Kim, M. K.; Kim, D. H.; Chang, I. S. *Bull. Korean Chem. Soc.* **2008**, *29*, 43.
11. Kim, D. H.; Hwang, J. S.; Baek, H. S.; Kim, K. J.; Lee, B. G.; Chang, I. S.; Kang, H. H.; Lee, O. S. *Chem. Pharm. Bull.* **2003**, *51*, 113.
12. Okombi, S.; Rival, D.; Bonnet, S.; Mariotte, A. M.; Perrier, E.; Boumendjel, A. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2252.
13. Sato, K.; Takahashi, H.; Iraha, R.; Toriyama, M. *Biol. Pham. Bull.* **2008**, *31*, 33.
14. Chun, H. J.; Jeong, S. I.; Woo, W. H.; Kim, I. K. *Bull. Korean Chem. Soc.* **2001**, *22*, 1159.
15. Guo, W.; Li, J.; Fan, N.; Wu, W.; Zhou, P.; Xia, C. *Synth Commun.* **2005**, *35*, 145.
16. Mosmann, T. *J. Immunol. Meth.* **1983**, *65*, 55.
17. Russell, C. A.; Vindelov, L. L. *J. Immunol. Meth.* **1998**, *217*, 165.
18. Masamoto, Y.; Lisa, K.; Kubo, M. *Planta. Med.* **1980**, *40*, 361.
19. Lee, H. S. *J. Agric. Food Chem.* **2002**, *50*, 1400.
20. Kong, K. H.; Park, S. Y.; Hong, M. P.; Cho, S. H. *Comp. Biochem. Physiol. B* **2000**, *125*, 563.
21. Ko, F. N.; Liao, C. H.; Kuo, Y. H.; Lin, Y. L. *Biochem. Biophys. Acta* **1995**, *1258*, 145.
22. Kim, B. T.; O, K. J.; Chun, J. C.; Hwang, K. J. *Bull. Korean Chem. Soc.* **2008**, *29*, 1125.
23. Shimizu, K.; Fukunaga, S.; Yoshikawa, K.; Kondo, R. *J. Wood. Sci.* **2006**, *53*, 153.