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# Synthesis of tyrosinase inhibitory (4-oxo-4*H*-pyran-2-yl)acrylic acid ester derivatives

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ABSTRACT

Melanogenesis is a physiological process that results in the production of melanin pigment. However, excessive accumulations of epidermal pigmentation can cause various hyperpigmentary disorders such as, melasma and age spots. Kojic acid and hydroxylated cinnamic acid derivatives are known to inhibit tyrosinase, a key component of melanin biosynthesis. Pyronyl-acrylic acid esters **3a–i**, which share structural features of kojic acid and hydroxylated cinnamic acid, were prepared and their abilities to inhibit tyrosinase and melanin production were evaluated. Of the esters synthesized, **3e** and **3h**, which derived from diethylene glycol moieties were found to inhibit melanin production by *ca.* 20% at 20 μg/ml, whereas kojic acid at 200 μg/ml inhibited melanin production by 15.8%.



Melanogenesis is a physiological process that results in the production of melanin pigment, which plays an important role in the prevention of sun-induced skin injuries. Although melanin production in human skin represents a primary defense mechanism against UV light, excessive accumulations of epidermal pigmentation can cause various hyperpigmentation disorders, such as, melasma, age spots, and sites of actinic damage. Tyrosinase (EC 1.14.18.1), a copper-containing enzyme, catalyzes two distinct reactions during the biosynthesis of melanin, i.e., the hydroxylation of L-tyrosine to L-dopa and the oxidation of L-dopa to dopaquinone, which is highly reactive and can polymerize spontaneously to form melanin.[1] Therefore, the regulation of melanin synthesis via the inhibition of tyrosinase is a current research topic in the context of preventing hyperpigmentation.[2]

Kojic acid (**1**) is produced by various fungi and bacteria, such as, *Aspergillus* and *Penicillium*, and is widely used as a skin-whitening agent because it inhibits tyrosinase.[3] However, its inhibitory effect and storage properties are inadequate for use in cosmetics. Accordingly, many semi-synthetic kojic acid derivatives have been synthesized, usually by modifying the C-7 hydroxyl group to form esters[4] or hydroxyphenyl ethers[5] or by using this group to form glycosides[6] or peptide[7] derivatives. Previously, we reported the synthesis of the kojic acid derivative bis-(4-oxo-4*H*-pyran-2-yl)ethene,[8] and have described the isolation of flavonoids, which are potent tyrosinase inhibitors, from *Glycyrrhiza uralensis*.[9]

Hydroxylated cinnamic acid derivatives, such as, caffeic and ferulic acids are common in plant cells and have been reported to inhibit tyrosinase.[10] Furthermore, amide derivatives of these acids have been isolated from plants[11] or synthesized and examined with respect to their abilities to inhibit human melanoma-tyrosinase.[12] In this study, we designed a novel compound by combining structures of two putative tyrosinase inhibitors, kojic acid (**1**) and caffeic acid (**2**) to form **3**. Although many kojic acid derivatives have been synthesized for tyrosinase inhibitors by the attachment of suitable groups to C-7 hydroxyl group,[4–7] reports on the structural modification of kojic acid skeleton are scarce. Herein, we describe the syntheses of pyronyl-acrylic acid esters **3a–i**, which posses a copper-chelating pyrone ring and the conjugated double bond present in caffeic acid (Fig. 1).

Since ester derivatives of kojic acid[13] and caffeic acid[14] have different abilities to inhibit tyrosinase. In this study, we synthesized ester derivatives of pyronyl-acrylic acid using its acid as a functional group. Hydroxyethyl, methoxyethyl, and their oligo analogs, which can be effective in increasing water solubility of compounds,[15] were also synthesized and their activities investigated.

Pyronyl-acrylic acid esters **3a–g** were synthesized using the Horner-Emmons reaction between aldehyde **4** and phosphonate **5**. PMB-protected comenaldehyde **4**[9] was condensed with **5**

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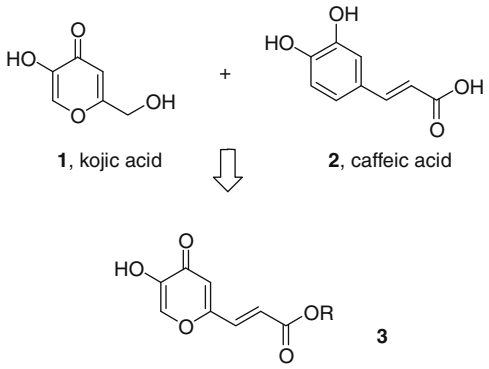



**Fig. 1.** Design of the New tyrosinase inhibitors.

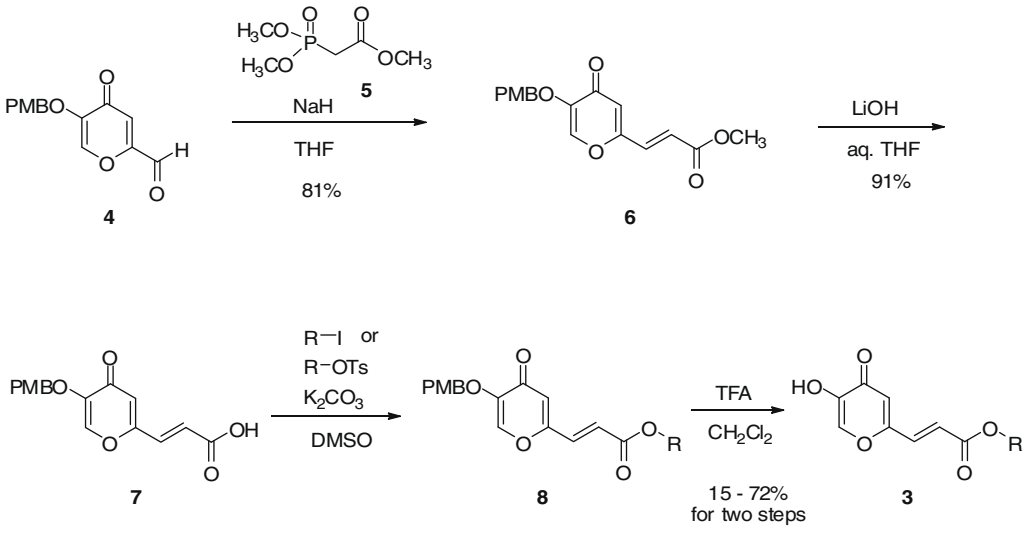
using NaH in THF to give the PMB-protected methyl ester **6** (Scheme 1). To synthesize different esters, the methyl ester group in **6** was hydrolyzed, using LiOH in aqueous THF, to give the pyronyl-acrylic acid **7**. Compound **7** was then alkylated with alkyl iodides to form **3a**–**c** using $K_2CO_3$ as a base. Esters containing ethylene glycol ether units (**3d**–**i**) were prepared by treating **7** with corresponding alkyl tosylates.[16] Finally, the PMB-protecting group in **8** was hydrolyzed using trifluoroacetic acid (TFA) in $CH_2Cl_2$ to provide the pyronyl-acrylic acid esters **3a**–**i** in 15∼72% overall yields from **7**.[17] In case of using hydroxyalkyl tosylate for the syntheses of **3g**–**i**, the overall yields were relatively low in 15–31% probably because of the involvement of hydroxyl group during the coupling and deprotection steps leading to the side reactions.

The resulting pyronyl-acrylic acid esters **3a–i** were assayed in terms of their abilities to inhibit tyrosinase and the productions of melanin. Results are summarized in Table 1.[18–20] Activity data for kojic acid are included for comparison purposes. The inhibitory effects of the synthesized compounds were also examined on melanin production and their cytotoxicities on B16F10 mouse melanoma cells at concentrations of 20 and 40 μg/ml. However, because kojic acid is inactive at these concentrations, its melanin production inhibiting and cytotoxic effects were evaluated at 200 and 400 μg/ml.

The ethyl ester **3a** inhibited tyrosinase at a level similar to that of kojic acid, with an $IC_{50}$ value of 36.3 μM. When the size of the alkyl group at ester was increased (**3b**), inhibitory activity was decreased, and no inhibition was observed at 100 μM when a long chain was introduced as in **3c**. Compounds **3a** and **3b** inhibited melanin production by 46.1% and 49.5%, respectively, at concentrations of 20 μg/ml. However, inhibitions by these compounds appeared to be due in part to their cytotoxic effects. On the other hand, compound **3c** inhibited melanin production by 25% and was less cytotoxic (85% of melanoma cells survived at 20 μg/ml). However, **3c** did not inhibit tyrosinase under concentration of 400 μM, which suggested that some other mechanism is responsible for inhibiting melanin production.

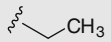
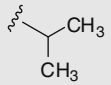
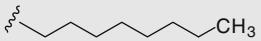
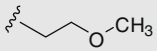
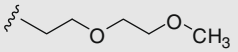
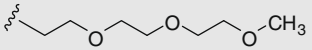
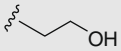
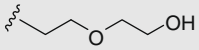
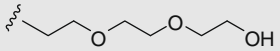
When the ethylene glycol moieties were incorporated through the ester group, a clear structure–activity relationship on the melanin production inhibitory activity was found although the synthesized compounds exhibited slightly less potent tyrosinase inhibitory activities than kojic acid. Increased number of an ethylene glycol or ethylene glycol methyl ether moiety in the esters decreased melanin production inhibitory activity (potencies; **3d** → **3f**, **3g** → **3i**). Compounds **3d** and **3g** (the esters of mono ethylene glycol moieties) inhibited melanin production in 29.6% and 30.0% at 20 μg/ml, respectively, and were substantially more effective than kojic acid (15.8% inhibition at 200 μg). In terms of cytotoxicity and tyrosinase and melanin production inhibition, compounds **3e** and **3h**, which possess diethylene glycol moieties, were best, i.e., they inhibited melanin production by ∼20% with melanoma cells survival of ∼80% at 20 μg/ml.

In conclusion, the pyronyl-acrylic acid esters **3a–i**, combined structures of kojic acid and caffeic acid, were synthesized and examined in terms of their abilities to inhibit melanin synthesis. Of the various esters synthesized, compounds **3e** and **3h**, possessing diethylene glycol moieties, were found to inhibit melanin production by ∼20% and to leave >80% of melanoma cells at 20 μg/ml, whereas the putative tyrosinase inhibitor, kojic acid, inhibited melanin production by 15.8% at 200 μg/ml at a cell viability of 79.9%. These results suggest that the hybridization of the structures of kojic acid and caffeic acid can lead to more potent inhibitors of melanin production.




**Scheme 1.**

**Table 1**
Chemical yields, tyrosinase and melanin synthesis inhibitory activities and cytotoxicities of **3a–i**.

| Compound | R | Yields (%) from **7** | Tyrosinase inhibition, $IC_{50}$ (μM) | % Inhibition of melanin production | | % Cell viability[a] | |
|---|---|---|---|---|---|---|---|
| | | | | 20 μg/ml | 40 μg/ml | 20 μg/ml | 40 μg/ml |
| **3a** |  | 72 | 36.3 | 46.1 | 65.0 | 72.0 | 39.2 |
| **3b** |  | 45 | 80.3 | 19.5 | 69.8 | 50.9 | 37.4 |
| **3c** |  | 63 | >400 | 25.0 | 54.6 | 85.0 | 62.5 |
| **3d** |  | 60 | 98.1 | 29.6 | 47.8 | 81.1 | 65.4 |
| **3e** |  | 52 | 59.1 | 19.5 | 24.0 | 88.7 | 83.4 |
| **3f** |  | 64 | 67.6 | 11.0 | 25.4 | 83.9 | 79.7 |
| **3g** |  | 31 | 74.9 | 30.0 | 41.6 | 80.5 | 74.8 |
| **3h** |  | 21 | 26.5 | 20.1 | 32.4 | 84.6 | 89.4 |
| **3i** |  | 15 | 79.2 | 8.7 | 17.9 | 84.9 | 78.5 |
| **1** | Kojic acid | — | 32.3 | 15.8[b] | 31.1[c] | 79.9[b] | 79.1[c] |

[a] % survivals of B16F10 melanoma cells.
[b] Tested at 200 μg/ml.
[c] Tested at 400 μg/ml.

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17. The spectral data of selected final compounds: **3e:** $^1$H NMR ($CDCl_3$) δ 7.87 (s, 1H, pyrone-*H*-6), 7.26 (d, 1H, *J* = 15.9 Hz, —*CH*═CH—$CO_2$—), 6.69 (d, 1H, *J* = 15.9 Hz, —CH═*CH*—$CO_2$—), 6.56 (s, 1H, pyrone-*H*-3), 4.40–3.58 (m, 8H, —O*CH*$_2$*CH*$_2$O—), 3.39 (s, 3H, —O*CH*$_3$), **3h:** $^1$H NMR ($CDCl_3$) δ 7.87 (s, 1H, pyrone-*H*-6), 7.27 (d, 1H, *J* = 15.6 Hz, —*CH*═CH—$CO_2$—), 6.74 (d, *J* = 15.6 Hz, 1H, —CH═*CH*—$CO_2$—), 6.58 (s, 1H, pyrone-*H*-3), 4.40–3.84 (m, 8H, —O*CH*$_2$*CH*$_2$O—).
18. *Mushroom tyrosinase inhibition assay:* Tyrosinase activity was determined using a method described by Tomita et al.[21] with slight modification; kojic acid was used as the positive control. Briefly, 50 μl of 0.1 M phosphate buffer (pH 6.8) was added to a 96-well plate, and to this was added 50 μl of L-tyrosine solution (0.3 mg/ml in water), 5 μl of tyrosinase (Sigma, 2 U/μl in buffer), and 40 μl of water pre-mixed in a micro-tube. 5 μl of test substance was then added (B) and incubated at 37° C for 10 min. The amount of dopa produced in reaction mixtures were measured at 475 nm. Sample inhibitory activities were expressed as concentrations that inhibited 50% of the enzyme activity ($IC_{50}$). The same solution without test substance (A) was also prepared. UV absorbances were measured at 475 nm, and % inhibitions were calculated using $[(A - B)/A] \times 100$.
19. *Determination of melanin contents:* Melanin contents were determined as described by Hosoi et al.[22] with slight modification, in triplicate, at least twice. On day 1, a total of $8 \times 10^4$ cells were added to 60 mm plates, and incubated at 37 °C in a 5% $CO_2$ incubator. On day 2, 10 μl test samples in DMSO were added to plates and incubated for 72 h at 37 °C in a $CO_2$ incubator. After washing with PBS, cells were lysed with 1 ml of 1N NaOH, and 200 μl portions of crude cell extracts were transferred to 96-well plates. Melanin contents were determined at 405 nm. The effects of test samples on melanin contents are expressed as percent inhibitions of the value obtained in B16F10 mouse melanoma cells cultured with DMSO alone (control).
20. *MTT assay:* MTT assays were performed using a micro-culture MTT method.[23] Briefly, a B16F10 mouse melanoma cell suspension was poured into a 96-well plate ($10^3$ cells/well) and cells were allowed to completely adhere overnight. Test samples were then added to the plate and incubated at 37 °C for 72 h in a $CO_2$ incubator. Twenty microliters of MTT solution (2 mg/ml) was then added per well and incubated for 4 h. Supernatant was then removed and formazan was solubilized by adding 150 μl DMSO to each well with gentle shaking. Optical densities of resulting supernatants were measured at 540 nm using an ELISA reader (Molecular Devices 09090, USA).
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