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Notes

Synthesis of Dimers of (4-Oxo-4*H*-pyran-2-yl)acrylic Acid as Tyrosinase Inhibitors

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Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme which catalyzes two distinct reactions in the biosynthesis of melanin; the hydroxylation of L-tyrosine to L-dopa and the oxidation of L-dopa to dopaquinone. The dopaquinone is highly reactive and can polymerize spontaneously to form melanin in a series of reaction pathway.[1] Therefore, the regulation of melanin synthesis *via* the inhibition of tyrosinase has been recent subject of many studies to prevent hyperpigmentation.[2]

Kojic acid (**1**) is one of metabolites produced by various fungal or bacterial strains, such as, *Aspergillus* and *Penicillium* and has been widely used in many countries as a skin-whitening agent because of its tyrosinase inhibitory activity.[3] However, its inhibitory activity for melanin synthesis or storage properties are not sufficient for use in cosmetics. Accordingly, many semi-synthetic kojic acid derivatives were synthesized by the modification of C-7 hydroxyl group into esters,[4] hydroxyphenyl ethers,[5] glycosides,[6] amino acid derivatives,[7] and tri-peptides.[8] We have also reported potent tyrosinase inhibitory bis-(4-oxo-4*H*-pyran-2-yl)ethene[9] and (4-oxo-4*H*-pyran-2-yl)propenic acid esters (**2**)[10] as kojic acid derivatives. In our previous studies, the (4-oxo-4*H*-pyran-2-yl)acrylic acid esters (**2**) were designed as combined structures of putative tyrosinase inhibitor kojic acid (**1**) and cinnamic acid derivatives and found to have good tyrosinase and melanin synthesis inhibitory activities when compared to that of kojic acid (**1**).

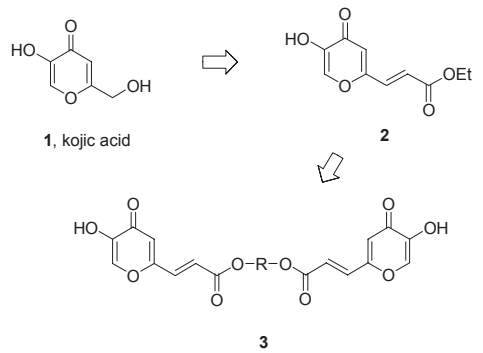
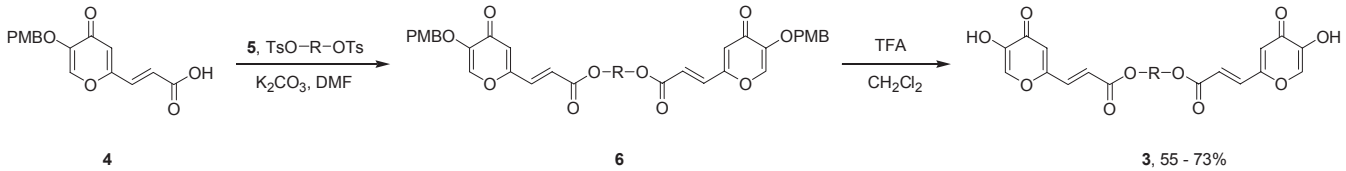


Figure 1. Design of new tyrosinase inhibitors.

The C_2 symmetric compounds are widely abundant in nature and some of them have interesting biological activities.[11] Furthermore, the dimerization of a pharmacophore of bioactive compounds through a linker is a well-known strategy for obtaining improved activity.[12-14] In this regard, we designed dimeric structures **3**, which have two (4-oxo-4*H*-pyran-2-yl)acryloyl groups connected through ester bonds as shown in Figure 2. Mono- and di(ethylene glycol) and propylene glycol were used as a linker to examine the effect of ether on the activity.

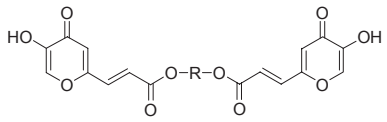
The synthesis of dimers **3a-d** was accomplished by coupling of pyronyl-acrylic acid **4** with the corresponding tosylates **5** and is summarized in Scheme 1. The di-tosylates **5** are commercially available or were readily prepared according to a reported procedure from the reaction of corresponding glycols with excess tosyl chloride in pyridine.[15] The 2.5 equivalents of PMB-protected pyronyl-acrylic acid **4**, which was derived from kojic acid,[10] was reacted with di-tosylates **5** to give **6**, PMB-protected pyronyl-acrylic acid dimers. The PMB-protecting group in **6** was hydrolyzed by treatment of trifluoroacetic acid (TFA) in CH_2Cl_2 to provide **3a-d** in 55 - 73% yields for two steps from di-tosylates **5**.

The resulting pyronyl-acrylic acid dimers **3a-d** were assayed on inhibition of tyrosinase and melanin production, and the results are summarized in Table 1.[16,17] The activity data of kojic acid and the pyronyl-acrylic acrylic acid ester **2** were included as standards for comparison. The synthesized compounds were also examined on their cytotoxicites by measuring cell viability

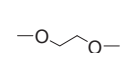
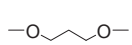
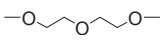
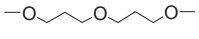
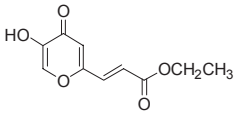


Scheme 1

Table 1. Chemical yields, tyrosinase and melanin synthesis inhibitory effects of **3a~d**.



3a~3d

Compounds	−O-R-O−	Yields (%) from **5**	Tyrosinase Inhibition, IC_{50} (μM)	% Inhibition of melanine synthesis[a]	
				at 10 μg/mL	at 20 μg/mL
3a		55	94.8	25.8 (56.1)	17.0 (46.0)
3b		73	34.5	25.6 (85.3)	33.6 (72.1)
3c		72	59.8	15.1 (85.6)	24.7 (82.4)
3d		61	33.6	22.4 (86.1)	26.8 (80.5)
1	Kojic acid	-	32.3	14.3 (88.4)[b]	15.8 (79.9)[c]
2		-	36.3	29.7 (75.3)	46.1 (62.0)

[a]In the parenthesis are % survival of B16F10 melanoma cells, [b]% survival at 100 μg/mL, [c]% survival at 200 μg/mL.

using B16F10 melanoma cell at two doses, 10 and 20 μg/mL concentrations, respectively. Since kojic acid was inactive at these concentrations, its melanin production inhibition activity and cytotoxicity were evaluated at 100 and 200 μg/mL.

The pyronyl-acrylic acid dimers **3** exhibited tyrosinase inhibitory activities at the similar level to that of corresponding monomer, pyronyl-acrylic acid ethyl ester **2**. They showed better inhibitory activities than kojic acid (**1**) and similar to lesser activities than **2** on the production of melanin in melanoma cells. On the other hands, every compound except **3a** showed lower cytotoxicities than **2** indicating that the selection of a suitable linker is also important on the cytotoxicity. In the aspects of inhibition of tyrosinase and melanin production, and cytotoxicity, compound **3d**, which have di(propylene glycol) as a linker, was the best compounds, which exhibited 22.4% inhibitions of melanin production while shows 86% melanin cells survivals at 10 μg/mL.

In summary, dimers of pyronyl-acrylic acid **3a-d** were prepared as potent inhibitors of melanin synthesis by coupling of pyronyl-acrylic acid with di-tosylates. They showed good inhibition on melanin production with lower cytotoxicities than parent compound, pyronyl-acrylic acid ester **2**. Among synthesized, compound **3d** having a di(propylene glycol) as a linker showed 22.4% inhibitions of melanin production with 86% survival of melanin cells at 10 μg/mL while kojic acid shows 14.3% inhibition of melanin production at 100 μg/mL.

Experimental Section

General. ^{1}H NMR spectra were recorded on a Gemini Varian-200 (200 and 50 MHz, respectively). Analytical thin layer chromatographies (TLC) were carried out by precoated silica gel (E. Merck Kiesegel $60F_{254}$, layer thickness 0.25 mm). All solvents used were purified according to standard procedures.

General procedure for the synthesis of dimmers 3a-d: To a solution of di-tosyl ether[14] (0.2 mmol) and K_2CO_3 (0.6 mmol) in DMF (5 mL) was added 3-(5-hydroxy-4-oxo-4*H*-pyran-2-yl)-acrylic acid (0.5 mmol) and the mixture was heated at 80 °C for 2 ~ 5 h. After checking the disappearence of di-tosyl ether by TLC, the mixture was cooled to rt, diluted with ethyl acetate (30 mL), and poured into cold water (30 mL). The organic layer was washed with water (30 mL × 2), dried over anhydrous $MgSO_4$ and evaporated. The residue was recrystallized from ethyl acetate and *n*-hexane to give PMB-protected dimmers **6a-d** in pure form. These compounds were dissolved in CH_2Cl_2 (10 mL) and treated with trifluoroacetic acid (1.0 mmol). After stirring at rt for 3 ~ 6 h, the mixture was evaporated and the resulting solid was recrystallized from ethyl acetate and *n*-hexane to provide dimmers **3a-d** in pure form.

3-(5-Hydroxy-4-oxo-4*H*-pyran-2-yl)-acrylic acid 2-[3-(5-hydroxy-4-oxo-4*H*-pyran-2-yl)-acryloyloxy]-ethyl ester (3a): Treatment of ethylene glycol di-tosylate with **4** according to general procedure provided the desired product **3a**. Yield 55%.

^{1}H-NMR (DMSO) δ 8.08 (s, 2H, pyrone-H-6), 7.42 (d, 2H, J = 15.9 Hz, -CH=CH-CO_2-), 6.85 (s, 2H, pyrone-H-3), 6.59 (d, 2H, J = 15.9 Hz, -CH=CH-CO_2-), 4.43 (s, 4H, -$CO_2CH_2OCH_2CO_2$-).

3-(5-Hydroxy-4-oxo-4*H*-pyran-2-yl)-acrylic acid 3-[3-(5-hydroxy-4-oxo-4*H*-pyran-2-yl)-acryloyloxy]-propyl ester (3b): Treatment of propylene glycol di-tosylate with **4** according to general procedure provided the desired product **3b**. Yield 73%. ^{1}H-NMR (DMSO) δ 8.07 (s, 2H, pyrone-H-6), 7.39 (d, J = 15.9, 2H, -CH=CH-CO_2-), 6.83 (s, 2H, pyrone-H-3), 6.58 (d, J = 15.9, 2H, -CH=CH-CO_2-), 4.26 (m, 4H, -$CO_2CH_2CH_2CH_2CO_2$-), 2.02 (m, 2H, -$CO_2CH_2CH_2CH_2CO_2$-).

3-(5-Hydroxy-4-oxo-4*H*-pyran-2-yl)-acrylic acid 2-{2-[3-(5-hydroxy-4-oxo-4*H*-pyran-2-yl)-acryloyloxy]-ethoxy}-ethyl ester (3c): Treatment of di(ethylene glycol) di-tosylate with **4** according to general procedure provided the desired product **3c**. Yield 72%. ^{1}H-NMR (DMSO) δ 8.05 (s, 2H, pyrone-H-6), 7.38 (d, 2H, J = 15.9 Hz, -CH=CH-CO_2-), 6.83 (s, 2H, pyrone-H-3), 6.58 (d, 2H, J = 15.9 Hz, -CH=CH-CO_2-), 4.28 (t, 4H, -$CO_2CH_2CH_2OCH_2CH_2CO_2$-), 3.70 (t, 4H, -$CO_2CH_2CH_2OCH_2CH_2CO_2$-).

3-(5-Hydroxy-4-oxo-4*H*-pyran-2-yl)-acrylic acid 3-{3-[3-(5-hydroxy-4-oxo-4*H*-pyran-2-yl)-acryloyloxy]-propoxy}-propyl ester (3d): Treatment of di(propylene glycol) di-tosylate with **4** according to general procedure provided the desired product **3d**. Yield 61%. ^{1}H-NMR ($CDCl_3$) δ 7.89 (d, 2H, pyrone-H-6), 7.25 (m, 2H, -CH=CH-CO_2-), 6.60-6.71 (m, 4H, pyrone-H-3 and -CH=CH-CO_2-), 3.37-4.20 (m, -CO_2CH_2- and -CH_2O-), 1.12-1.30 (m, -$CH_2CH_2CH_2$- and -$CH(CH_3)CH$-).

Mushroom tyrosinase inhibition assay. Tyrosinase activity was determined by the method described by Tomita *et al*.[16] with slight modification and kojic acid was used as a positive control. Briefly, to a 96-well plate was added 0.1 M phosphate buffer (pH 6.8) 50 μL, 50 μL of L-tyrosine solution (0.3 mg/mL in water), 5 μL of tyrosinase (Sigma, 2 units/μL in buffer), and 40 μL of water were mixed in a micro-tube, and then 5 μL of the test substance were added (B). After incubation at 37 °C for 10 min, the amount of dopa produced in the reaction mixture was measured at 475 nm. The inhibitory activity of sample was expressed as the concentration, which inhibits 50% of the enzyme activity (IC_{50}). The same solution without test substance (A) was also prepared and the UV absorbance was measured at 475 nm. The % inhibition was calculated using the formula [(A − B)/A] × 100.

Cell culture. B16F10 mouse melanoma cells were purchased from the American Type Culture Collection. The cells were grown in DMEM (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, USA) and penicillin/streptomycin (100 I.U./mL and 100 μg/mL, respectively, Sigma). The cells were maintained in a humidified incubator with 5% CO_2 at 37 °C. All tested compounds were prepared in DMSO.

Determination of melanin contents. Melanin contents were determined according to Hosoi *et al*.[17] with modification and performed in triplicate at least twice. On day 1, a total of 8×10^4 cells were added to 60 mm plates, and were incubated at 37 °C in 5% CO_2 incubator. On day 2, each 10 μL of test samples in DMSO were added to the plate, which was then incubated at 37 °C for 72 h in a CO_2 incubator. After being washed with PBS, the cells were lysed with 1 mL of 1 N NaOH, and 200 μL portions of crude cell extract were transferred to 96-well plates. Melanin content was determined at 405 nm. Effect of the test samples on melanin content was expressed as the percent inhibition of the value obtained in B16F10 mouse melanoma cells cultured with DMSO alone (control).

MTT assay. MTT assay was performed according to a microculture MTT method.[18] Briefly, B16F10 mouse melanoma cell suspension was poured into a 96-well plate (10^3 cells/well) and the cells were allowed to completely adhere to the plate overnight. Then, each test samples were added to the plate, which was then incubated at 37 °C for 72 h in a CO_2 incubator. After incubating, 20 μL of MTT solution (2 mg/mL) was added to each well and incubated for 4 h and then supernatant was removed. The formazan dye was solubilized by adding 150 μL DMSO to each well, followed by gentle shaking. The optical density of the resulting supernatant was measured at 540 nm using an ELISA reader (Molecular Devices 09090, USA).

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