Full Paper

Synthesis of Tyrosinase Inhibitory Kojic Acid Derivative

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Kojic acid derivative **2** was synthesized by joining two pyrone rings through an ethylene linkage by Horner-Emmons reaction of phosphonate **6** with aldehyde **7**. The intermediates **6** and **7** were derived from kojic acid. The tyrosinase inhibitory activity of **2** was about 8 times more potent (IC_{50} = 3.63 μM) than that of kojic acid (IC_{50} = 30.61 μM). Compound **2** also exhibited potent melanin synthesis inhibitory activity (19.53% inhibition at 5 μg) indicating that the connection of two pyrone rings of kojic acid through a suitable linker can be an useful strategy for identification of potent tyrosinase inhibitiors.

Keywords: Tyrosinase inhibitor / Kojic acid / Melanin production / Skin-whitening

Received: September 25, 2005; accepted: October 21, 2005

DOI 10.1002/ardp.200500213

Introduction

Melanogenesis is a physiological process resulting in the production of melanin pigment, which plays an important role in the prevention of sun-induced skin injury [1]. Although the melanin production in human skin is a major defense mechanism against UV light, the accumulation of an excess of epidermal pigmentation can cause various hyperpigmentation disorders, such as melasma, age spots, and sites of actinic damage.

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme widely distributed in nature. It catalyzes two distinct reactions involving molecular oxygen in the melanin synthesis, the hydroxylation of L-tyrosine to L-dopa and the oxidation of L-dopa to dopaquinone. This dopaquinone is highly reactive and can polymerize spontaneously to form melanin in a series of reaction pathways [2]. Accordingly, the regulation of melanin synthesis by inhibition of tyrosinase to prevent hyperpigmentation has been a recent subject of many studies [3].

Kojic acid (**1**, Fig. 1) is one of the metabolites produced by various fungal or bacterial strains such as *aspergillus* and *penicillium* and has been used in many countries as a skin-whitening agent because of its tyrosinase inhibitory activity on melanin synthesis [4]. However, the inhibitory activity of **1** is not sufficiently potent or unstable for storage for use in cosmetics. Accordingly, many semi-synthetic kojic acid derivatives were synthesized usually by the modification of C-7 hydroxyl group into ester [5] hydroxyphenyl ether [6], glycoside [7], and amide derivatives [8].

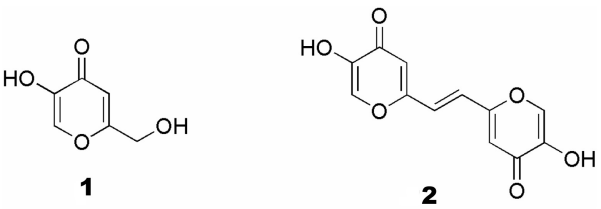

Figure 1. Chemical structure of kojic acid **1** and the newly synthesized derivative **2**.

In this study, we designed and synthesized a new kojic acid derivative **2** (Fig. 1), which has two pyrone rings connected by an ethylene linker. It was expected that compound **2** has better tyrosinase inhibitory activity with enhanced stability because it has two copper-chelating pyrone rings and a chemically stable ethylene rather than C-7 hydroxyl, ester or amide group.

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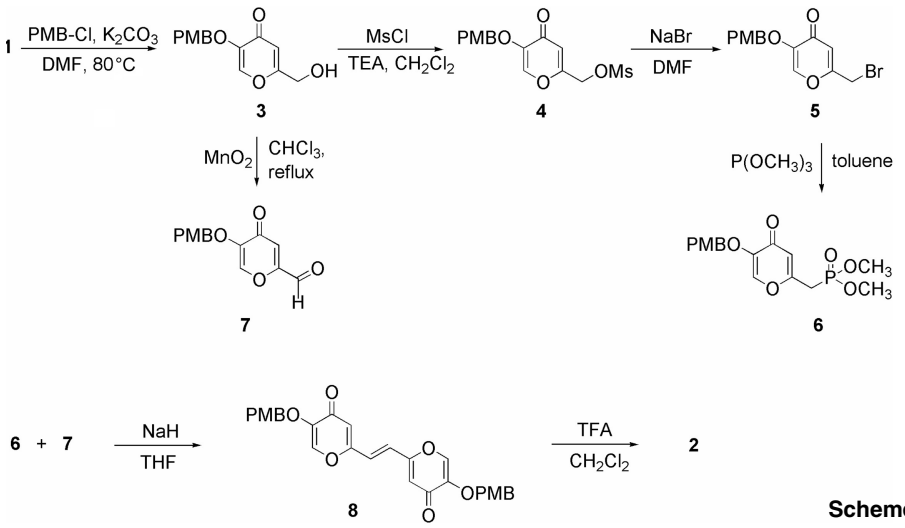

Scheme 1. Synthesis of kojic acid derivative.

Results and discussion

Chemistry

Kojic acid derivative **2** was synthesized by joining two pyrone rings by Horner-Emmons reaction of phosphonate with aldehyde components, both of which can be derived from kojic acid as shown in Scheme 1. The C-5 hydroxyl group of **1** was selectively protected with the reaction of *p*-methoxybenzyl chloride (PMB-Cl) to afford **3** in 81% yield [9]. The C-7 hydroxyl group of **3** was converted to bromide **5** via mesylation of alcohol followed by bromination of the resulting mesylate **4** with the reaction of sodium bromide in DMF in 58% yield for two steps [10]. Arbuzov reaction of **5** with trimethyl phosphite in toluene at reflux temperature gave phosphonate **6** in 79% yield.

For the preparation of aldehyde **7**, PMB-protected kojic acid **3** was used again. Compound **3** was oxidized with manganese oxide in chloroform to afford **7** in 75% yield. The Horner-Emmons reaction of phosphonate **6** with aldehyde **7** with NaH in DMF gave PMB-protected dimmer **8**, which was subjected to hydrolysis condition by treatment of trifluoroacetic acid (TFA) in CH_2Cl_2 to provide kojic acid derivative **2** in 78% yield.

Biological activity

The resulting kojic acid derivative **2** was assayed on inhibition of tyrosinase as shown in Table 1 and the activity data of kojic acid were included as standards for comparison. Compound **2** was also tested on the melanin synthesis using B16F10 melanoma cell to investigate the effects of tyrosinase inhibitory activities on melanin production. The cytotoxicities of **2** and kojic acid on melanoma cell were examined using MTT assay. The tyrosinase inhibitory activity of **2** was about 8 times more potent (IC_{50} = 3.63 μM) than that of kojic acid (IC_{50} = 30.61 μM). Compound **2** also exhibited superior melanin synthesis inhibitory activity (19.53% inhibition at 5 μg) at a nontoxic concentration compared to kojic acid (16.36% inhibition at 200 μg) indicating that joining of two pyrone rings of kojic acid through a suitable linker can be another strategy for identification of potent tyrosinase inhibitiors. However, further structural modification of **2** is needed for improvement of pharmacokinetic properties, since the solubility of **2** was low in working solution during assay procedures.

Table 1. Tyrosinase and melanin synthesis inhibitory effects of **2**.

Compound	Tyrosinase inhibition IC_{50}, [μM]	% Inhibition of melanin synthesis	% Survival of B16F10 melanoma cell
2	3.63	32.12 (at 12.5 μg)	93.38 (at 12.5 μg)
		19.53 (at 5 μg)	96.59 (at 5 μg)
		12.91 (at 2.5 μg)	98.50 (at 2.5 μg)
Kojic acid	30.61	16.37 (at 200 μg)	93.96 (at 200 μg)

This research was supported by a research grant of Kyung Hee University in 2004.

Experimental

Chemistry

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Gemini Varian-200 (200 and 50 MHz, respectively; Varian Inc., Palo Alto, CA, USA). Analytical thin layer chromatographies (TLC) were carried out by precoated silica gel (E. Merck Kiesegel $60F_{254}$ layer thickness 0.25 mm; Merck, Darmstadt, Germany). Flash column chro-

matographies were performed with Merck Kiesegel 60 Art 9385 (230 – 400 mesh). All solvents used were purified according to standard procedures.

5-(4-Methoxybenzyloxy)-2-hydroxymethyl-4H-pyran-4-one ***3***

To a stirred solution of kojic acid (**2**, 4.75 g, 33.4 mmol) and K_2CO_3 (9.68 g, 70.0 mol) in dry DMF (70 mL) was added PMB-Cl (5.66 mL, 41.7 mmol), then the reaction mixture was heated under refluxed for 1 h at 80°C. The solvent was removed under reduced pressure and the precipitate was filtered, washed with water and ethyl acetate to afford **3** as a white solid (7.04 g, 81%). ^{1}H-NMR (DMSO-d_6) δ 8.16 (s, 1H, pyrone-*H*6), 7.36 (d, 2H, *J* = 8.5 Hz, Ph-*H2,6*), 6.96 (d, 2H, *J* = 8.5 Hz, Ph-*H3,5*), 6.33 (s, 1H, pyrone-*H*3), 5.70 (t, 1H, *J* = 5.8 Hz, –OH), 4.87 (s, 2H, Ph–CH_2–), 4.30 (s, 2H, *J* = 5.8 Hz, –CH_2OH), 3.77 (s, 3H, OCH_3).

(5-(4-Methoxybenzyloxy)-4-oxo-4H-pyran-2-yl)methyl methanesulfonate ***4***

To a stirred solution of **3** (300 mg, 1.44 mmol) in CH_2Cl_2 (12 mL) was added triethylamine (0.19 mL, 1.37 mmol) and methanesulfonyl chloride (0.09 mL, 1.19 mmol) at 0°C. After stirring for 2 h at room temperature (r.t.), the mixture was diluted with EtOAc and washed with brine. The organic layer was dried by $MgSO_4$, concentrated, and solidified with ether and *n*-hexane to afford **4** as a white solid (312 mg, 80%). ^{1}H-NMR (DMSO-d_6) δ 8.28 (s, 1H, pyrone-*H6*), 7.35 (d, 2H, *J* = 8.5 Hz, Ph-*H2,6*), 6.95 (d, 2H, *J* = 8.5 Hz, Ph-*H3,5*), 6.58 (s, 1H, pyrone-*H3*), 5.15 (s, 2H, –CH_2OSO$_2$–), 4.87 (s, 2H, Ph–CH_2–), 3.76 (s, 3H, OCH_3), 3.31 (s, 3H, –SO$_2$CH_3).

5-(4-Methoxybenzyloxy)-2-bromomethyl-4H-pyran-4-one ***5***

To a stirred solution of **4** (300 mg, 0.88 mmol) in DMF (15 mL) was added NaBr (226 mg, 2.20 mmol) at r.t. After further stirring for 50 min, the reaction mixture was poured into water and extracted by EtOAc. The organic layer was dried over $MgSO_4$, concentrated, and solidified with ether and *n*-hexane to afford **5** as an ivory colored solid (194 mg, 72%). ^{1}H-NMR (DMSO-d_6) δ 8.25 (s, 1H, pyrone-*H6*), 7.35 (d, 2H, *J* = 8.5 Hz, Ph-*H2,6*), 6.96 (d, 2H, *J* = 8.5 Hz, Ph-*H3,5*), 6.57 (s, 1H, pyron-*H3*), 4.86 (s, 2H, Ph–CH_2–), 4.55 (s, 2H, -CH_2Br), 3.76 (s, 3H, OCH_3).

Dimethyl(5-(4-methoxybenzyloxy)-4-oxo-4H-pyran-2-yl)methylphosphonate ***6***

To a solution of **5** (300 mg, 0.92 mmol) in toluene (50 mL) was added trimethyl phosphite (1.08 mL, 9.22 mmol) and the mixture was heated at reflux. After 86 h, excess trimethyl phosphite was removed by simple distillation and the resulting oily residue was purified by flash column chromatography (CH_2Cl_2/CH_3OH = 20 : 1) to afford **6** as a white solid (258 mg, 79%). ^{1}H-NMR (DMSO-d_6) δ 8.18 (s, 1H, pyrone-*H6*), 7.33 (d, 2H, *J* = 8.6 Hz, Ph-*H2,6*), 6.98 (d, 2H, *J* = 8.6 Hz, Ph-*H3,5*), 6.32 (s, 1H, pyron-*H3*), 4.85 (s, 2H, Ph–CH_2–), 3.76 (s, 3H, -OCH_3), 3.65 and 3.70 (two s, each 3H, OCH_3), 3.42 (d, 2H, *J* = 21.6 Hz, CH_2PO–).

5-Hydroxy-4-oxo-4H-pyran-2-carbaldehyde ***7***

A mixture of **3** (4.0 g, 15.3 mmol) and manganese (IV) oxide (2.65 g, 30.6 mmol) suspended in $CHCl_3$ (100 mL) was heated at reflux for 12 h. An additional amount of manganese (IV) oxide (2.65 g, 30.6 mmol) was treated and the mixture was further stirred for 6 h. After cooling to r.t., the reaction mixture was filtered through Celite 545. The filtrate was concentrated and diluted with EtOAc. The insoluble solid was removed by filtration, again through Celite 545. The filtrate was concentrated and solidified with EtOAc and *n*-hexane to afford **7** as a light yellowish solid (3.0 g, 75%). ^{1}H-NMR ($CDCl_3$) δ 9.63 (s, 1H, –C*H*O), 7.66 (s, 1H, pyrone-*H*6), 7.31 (d, 2H, *J* = 8.51 Hz, Ph-*H2,6*), 6.97 (s, 1H, pyrone-*H*3), 6.89 (d, 2H, *J* = 8.51 Hz, Ph-*H3,5*), 5.05 (s, 2H, Ph–CH_2–), 3.80 (s, 3H, OCH_3); ^{13}C-NMR($CDCl_3$) δ 184.3, 174.6, 160.3, 156.0, 149.4, 142.1, 130.1, 127.5, 120.3, 114.5, 72.2, 55.6.

1,2-trans-Bis(5-hydroxy-4H-pyran-4-one-2-yl)ethene ***2***

To a stirred solution of **6** (200 mg, 0.56 mmol) and NaH (45 mg, 1.12 mmol) in dry THF (35 mL) was added a solution of **7** (131.1 mg, 0.50 mmol) in THF (5 mL) dropwise under N_2 atmosphere at 0°C. After stirring at r.t. for 1 h, the reaction mixture was poured into water. The resulting precipitate was filtered and washed with water and diethyl ether. The filtered cake was collected and dried *in vacuo* to give PMB-protected kojic acid derivative **8** as a white solid (226 mg, 83%), which was used in the next step without further purification. To a stirred solution of **8** (100 mg, 0.20 mmol) in CH_2Cl_2 (15 mL) was slowly added trifluoracetic acid (0.09 mL, 1.20 mmol) at r.t. The reaction mixture was stirred for 30 min and the solvent was removed by evaporation. The resulting solid was washed with diethyl ether and dried *in vacuo* to afford **2** as a yellow solid (48 mg, 94%). ^{1}H-NMR (DMSO-d_6) δ 8.11 (1H, s, pyrone-*H*2), 7.15 (1H, s, –C*H*=CH–), 6.72 (1H, s, pyrone-*H*5); ^{13}C-NMR (DMSO-d_6) δ 174.2, 159.1, 146.7, 139.9, 126.1, 115.1.

Biology

Mushroom tyrosinase inhibition assay

Tyrosinase activity was determined by the method described by Tomita *et al.* [11] with slight modification and kojic acid was used as a positive control. Briefly, to a 96-well plate was added 50 μL 0.1 M phosphate buffer (pH 6.8), 50 μL of L-tyrosine solution (0.3 mg/mL in water), 5 μL of tyrosinase (Sigma, 2×10^3 units/mL in buffer; http://www.Sigma-Aldrich.com) 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 $[(A-B)/A] \times 100$.

Cell culture

B16F10 mouse 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 (FBS, Gibco BRL) and penicillin/streptomycin (100 IU/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.* [12] 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 micro-culture MTT method [13]. 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 24 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 the 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 Corporation, Sunnyvale, CA, USA).

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