

New 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one Derivative Has Both Tyrosinase Inhibitory and Antioxidant Properties

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Kojic acid,¹ 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, is produced from carbohydrate sources in an aerobic process by a variety of microorganisms. It showed broad biological activities such as inhibition of tyrosinase,² scavenging of the free radicals,³ chelating activity of metal ions⁴ and prevention of photodamage.³ Its various activities are due to γ -pyranone structure having enolic hydroxyl group. Recently, enolic hydroxyl group of kojic acid has been focused as an alternative of carboxylic acid in retinoid structure.⁵ We synthesized 3,4-methylenedioxy cinnamic acid ester of kojic acid as a new retinoidal compound. In this study, we evaluated biological activities of new kojic acid derivative **1**, 2-((3*E*)-4(2*H*,3*H*-benzo[3,4-*d*]1,3-dioxolan-5-yl)-2-oxo-but-3-enyl-oxo)-5-hydroxy-4H-pyran-4-one.

Experimental Section

Synthesis. Compound **1** was synthesized by the condensation of kojyl chloride with potassium salt of 3,4-methylenedioxy cinnamic acid. Structures of compounds and synthetic pathways are shown in Figure 1. Kojic acid was reacted with thionyl chloride to afford a kojyl chloride **2**. Then, kojyl chloride **2** was reacted with potassium salt of 3,4-(methylenedioxy) cinnamic acid to afford the final compound **1**.

TLC, SiO₂, EtOAc/hexanes 2 : 1, R_f = 0.41 ¹H-NMR (300 MHz, DMSO-*d*₆) δ 9.20 (bs, 1H), 8.05 (s, 1H), 7.58 (d, 1H, *J* = 15.9 Hz), 7.39 (s, 1H), 7.19 (d, 1H, *J* = 8.4 Hz), 6.90 (d, 1H, *J* = 8.4 Hz), 6.55 (d, 1H, *J* = 15.9 Hz), 6.45 (s, 1H), 6.02 (s, 2H), 5.00 (s, 2H). IR ν_{max} (KBr) 3206, 1726 cm⁻¹. MS-FAB (*m/e*) 317 (*M*⁺+1).

Mushroom tyrosinase assay. Mushroom tyrosinase, L-tyrosine, and L-DOPA were purchased from Sigma Chemical (St. Louis, MO, USA). Tyrosinase activity was determined using the method of Pomerantz⁶ with minor modification. Twenty-five μ L of 0.5 mM L-DOPA, 25 μ L of 10 mM L-tyrosine, 875 μ L of 50 mM phosphate buffer (pH 6.5), and

25 μ L of test sample solution were mixed. Then 50 μ L of mushroom tyrosinase (1600 U/mL) was added. The amount of dopachrome produced in the reaction mixture was determined against a blank (solution without enzyme) at 475 nm (OD₄₇₅) using a spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

MTT growth assay. HaCaT keratinocytes were maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented 10% fetal bovine serum, previously inactivated at 56 °C for 20 min. The cytotoxic effects of test materials were monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as dose dependent manner.

Lipid peroxidation. HaCaT keratinocytes were grown in DMEM medium containing 10% fetal bovine serum and 1% antibiotic and antimycotic solution. For experiments, cells were maintained in DMEM supplemented with 1% fetal bovine serum (FBS) and test materials for 18 h. After HaCaT keratinocytes were incubated with test materials for 18 h, the cells were exposed to 4 mM *t*-BOOH for 4 h. Following incubation, the cell were washed twice with phosphate-buffered saline (PBS), and lysed by repetitive freeze/thawing in distilled water. To establish the levels of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxy-2(*E*)-nonenal (4-HNE) levels were quantified using a commercial colorimetric lipid peroxidation assay kit (Calbiochem, San Diego, CA). This method analyzes MDA and 4-HNE by their reaction with a chromogen (*N*-methyl-2-phenylindole) at 45 °C to produce a stable chromogen. The reaction products were measured by spectrophotometry at 586 nm. The procedure was performed in accordance with the manufacturer's specifications and data were expressed in mmol/mg protein.

Results and Discussion

Compound **1** is a kojic acid derivative which possesses an ester linker between kojic acid and 3,4-(methylenedioxy)-

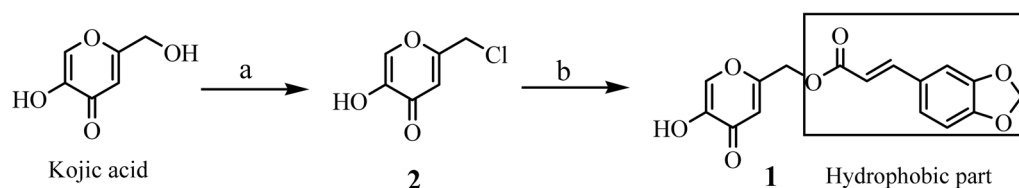


Figure 1. Reaction conditions; (a) SOCl₂, DMF; (b) Potassium salt of 3,4-(methylenedioxy)cinnamic acid, DMF.

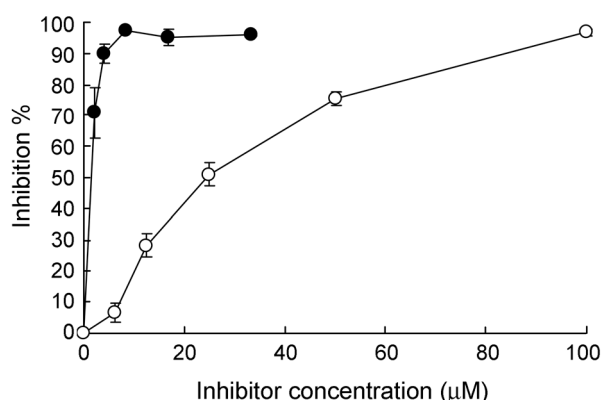


Figure 2. Dose-dependent inhibitory effects on mushroom tyrosinase by compound **1** and kojic acid. Samples shown are compound **1** (closed circle) and kojic acid (open circle). Effect on tyrosinase activity by samples as a function of concentration are represented as inhibition %, means \pm S.E. of the three independent tests.

cinnamate moiety. Cinnamate group was introduced as a hydrophobic moiety to increase tyrosinase inhibitory activity of kojic acid. The mushroom tyrosinase inhibitory activities of compound **1** and kojic acid were determined using L-tyrosine as substrate. When L-tyrosine was used as a substrate, compound **1** showed stronger inhibitory activity than that of kojic acid. IC_{50} of compound **1** is $1.4 \mu\text{M}$ (Fig. 2).

A kinetic study of L-tyrosine oxidation catalyzed by mushroom tyrosinase was accomplished in the presence of compound **1** and kojic acid (Fig. 3). Compound **1** and kojic acid showed the same Michaelis-Menten constant (K_m value). It means that the same moiety was used for inhibitory effects on the mushroom tyrosinase. Through Lineweaver-Burk plot data, compound **1** was a competitive inhibitor.

Another expected biological activity of compound **1** is an antioxidant effect. Recently, kojic acid showed inhibitory activity in lipid peroxidation.³ 5-Hydroxyl group of kojic acid is regarded as a hydrogen donor that results in radical scavenging activity. Cytotoxicity and inhibitory potency of compound **1** in lipid peroxidation was compared with known antioxidant agents such as trolox,⁷ EGCG⁸ and kojic acid. Cell viability was assessed by the MTT reduction assay. HaCat cells were resistant to up to $10 \mu\text{M}$ concentration for all test materials.

After confirming cell viability, we evaluated inhibitory activity of compound **1** and known antioxidants. Their activities were examined in terms of ability to reduce the oxidative factors such as malondialdehyde (MDA) and 4-hydroxy-2(*E*)-nonenal (4-HNE), generated by TBHP (*tert*-butylhydroperoxide) in HaCaT cell line.⁹ Treatment of 4 mM of TBHP increased lipid peroxide level up to about three times as compared with untreated sample. When $10 \mu\text{M}$ of compounds were treated, trolox, EGCG and compound **1** were active (Fig. 4). Compound **1** decreased the level of lipid peroxidation by about 47% in contrast with TBHP-treated control. However, kojic acid showed no inhibitory activity at $10 \mu\text{M}$ concentration.

Compound **1** showed more potent biological activities

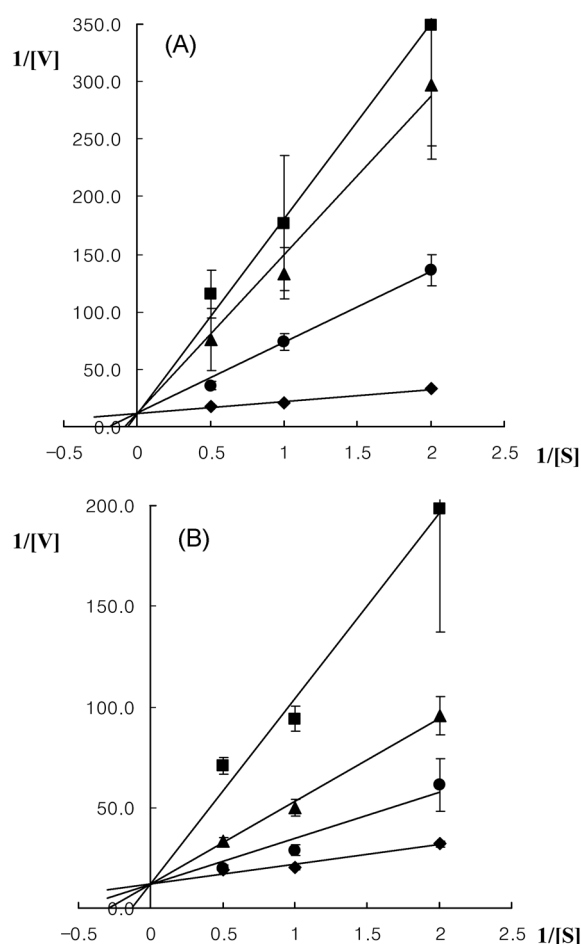


Figure 3. Lineweaver-Burk plot of mushroom tyrosinase on the presence of compound **1** and kojic acid. Data were obtained as mean value of $1/[V]$, inverse of the increase of optical density at 450 nm per min. (OD₄₅₀/min), of three independent tests with different concentrations of L-tyrosine as a substrate. (A) with $10 \mu\text{M}$ (rectangle), $5 \mu\text{M}$ (triangle), $2 \mu\text{M}$ (circle), or no compound **1** (diamond) and kojic acid (B) with $100 \mu\text{M}$ (rectangle), $50 \mu\text{M}$ (triangle), $20 \mu\text{M}$ (circle), or no kojic acid

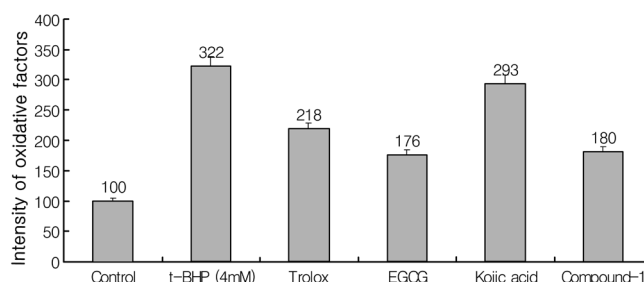


Figure 4. Inhibitory effects on lipid peroxidation induced by TBHP in HaCat cell line. All compounds were tested at $10 \mu\text{M}$ concentration.

than those of kojic acid in two tested methods. These results suggest that biological activities of kojic acid were increased by the addition of 3,4-(methylenedioxy)cinnamate moiety as a hydrophobic part. Kojic acid is hydrophilic compound because it has two hydroxyl groups in 2 and 5 positions. Compound **1** is believed to be more adequate in cell permeation than kojic acid because of its balance in hydrophilic

Table 1. Calculation of Log P values

Compound	Log P ^a
Kojic acid	-1.111
Compound 1	1.169

^aLog P: Log[octanol/water] partition coefficient

and hydrophobic character. To compare hydrophobic character of compound **1** with kojic acid, we calculated log P value (Table 1).

In conclusion, pharmacophore of kojic acid is enolic hydroxyl group in 5-position. To enhance biological activities of kojic acid, we increased hydrophobicity by introduction of 3,4-methylenedioxy cinnamate moiety in 2-position which is not pharmacophore. Its potent activities may be due to balance between hydrophilic and hydrophobic character.

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