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 ions[4] 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(2H,3H-benzo[3,4-d]1,3-dioxolan-5-yl)-2-oxo-but-3-enyloxy)-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, SiO_2, EtOAc/hexanes 2 : 1, R_f = 0.41 ^{1}H-NMR (300 MHz, DMSO-d_6) δ9.20 (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 Pomerantz[6] with minor modification. Twenty-five μL of 0.5 mM L-DOPA, 25 μL of 10 mM L-tyrosine, 875 μL of 50 mM phosphate buffer (pH 6.5), and 25 μL of test sample solution were mixed. Then 50 μL 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 (OD_{475}) 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)-

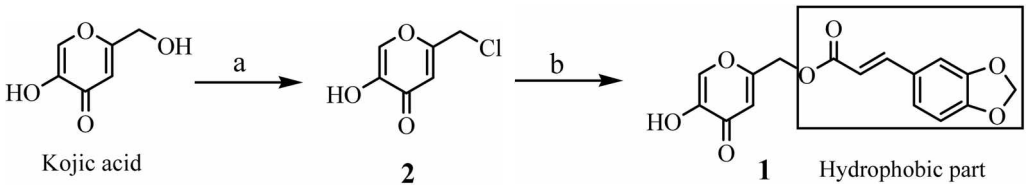

Figure 1. Reaction conditions; (a) $SOCl_2$, DMF; (b) Potassium salt of 3,4-(methylenedioxy)cinnamic acid, DMF.

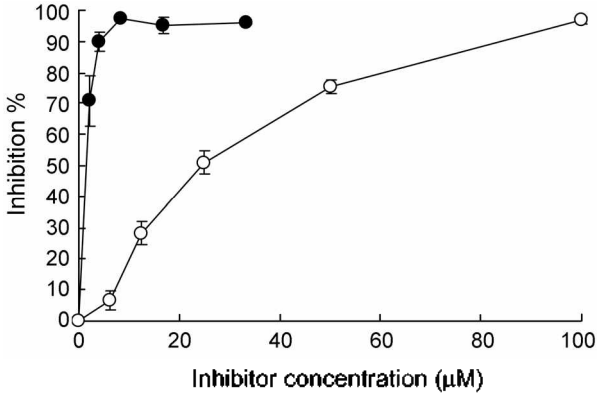

Figure 2. Dose-dependent inhibitory effects on mushroom tyrosinase by compound **1** and kojic acid. Samples shown are compound **1** (closed circle) and kojic acid (open circle). Effect on tyrosinase activity by samples as a function of concentration are represented as inhibition %, means ± S.E. of 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 μM (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] EGCG[8] and kojic acid. Cell viability was assessed by the MTT reduction assay. HaCat cells were resistant to up to 10 μM 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 μM 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 μM concentration.

Compound **1** showed more potent biological activities

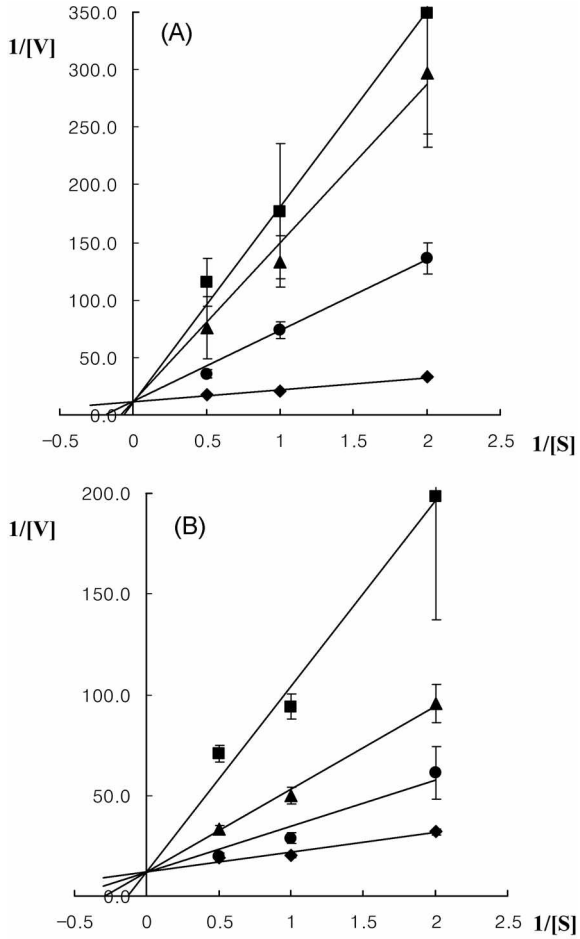

Figure 3. Lineweaver-Burk plot of mushroom tyrosinase on the presence of compound **1** and kojic acid. Data were obtained as mean value of 1/[V], inverse of the increase of optical density at 450 nm per min. (OD450/min), of three independent tests with different concentrations of L-tyrosine as a substrate.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

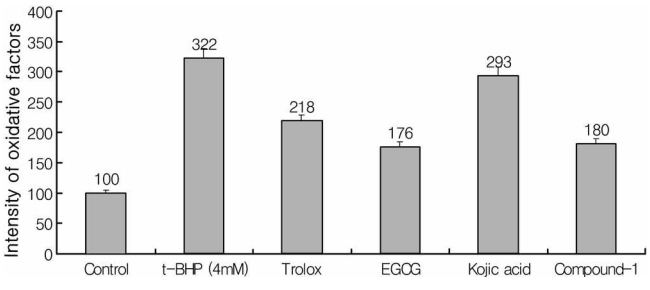

Figure 4. Inhibitory effects on lipid peroxidation induced by TBHP in HaCat cell line. All compounds were tested at 10 μM concentration.

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

[a]Log P: Log[octanol/water] partition coefficient

and hydrophobic character. To compare hydrophobic character of compound **1** with kojic acid, we calculated lop 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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