Synthesis of New Anti-melanogenic Compounds Containing Two Molecules of Kojic Acid

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Received March 24 , 2008

Key Words : Kojic acid derivative, Thioether, Tyrosinase inhibitor, Melan-a cell

Tyrosinase[1] is a copper-containing enzyme, which catalyzes two distinct reactions of melanin synthesis, the hydroxylation of L-tyrosine to L-dopa and the oxidation of L-dopa to dopaquinone. These processes are involved in local hyperpigmentation such as melasma[2] and lentigo.[3] Therefore, the inhibition of tyrosinase to treat the pigmentation disorders has been a recent subject of many studies. Kojic acid[4] (**1**) is well known as one of the most popular tyrosinase inhibitors and it has been widely used as a skin-whitening agent. However, the inhibitory activity of kojic acid is not potent enough. To overcome this drawback, many semi-synthetic kojic acid derivatives were synthesized usually by the modification of C-2 hydroxyl group into ester,[5] hydroxyphenyl ether,[6] and glycoside.[7] Recently, two molecules of kojic acid were connected by ester[8] and ethylene.[9] Their inhibitory activities were dramatically enhanced. Thus we became interested in new kojic acid derivatives containing two molecules of kojic acid. In this study, we connected two molecules of kojic acid by various chemical bonds such as ester, amide, and thioether. Anti-melanogenic activities of compounds (**4a-4f**) were compared with that of kojic acid (Figure 1).

Experimental Section

Synthesis

Preparation of 4a. To a stirred solution of kojyl chloride **2** (4.8 g, 30 mmol) in DMF (80 mL) under N_2 was added potassium salt of kojyl succinic acid (10.1 g, 36 mmol). The reaction mixture was stirred for 4 h at 110 °C. DMF was evaporated *in vacuo* and the residue was extracted with ethyl acetate (300 mL), washed with water. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate-hexane to give a **4a** (9.1 g) in 83% yields. R_f = 0.34 (SiO_2, 2:1 EtOAc/hexanes); ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.22 (bs, 2H), 8.06 (s, 2H), 6.46 (s, 2H), 4.96 (s, 4H), 2.71 (s, 4H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.88, 171.57, 161.63, 146.26, 140.09, 112.69, 61.67, 28.52. IR ν_{max} (KBr) 3404, 1726, 1653 cm^{-1}. FABMS: (m/e) 367 $[M+H]^+$.

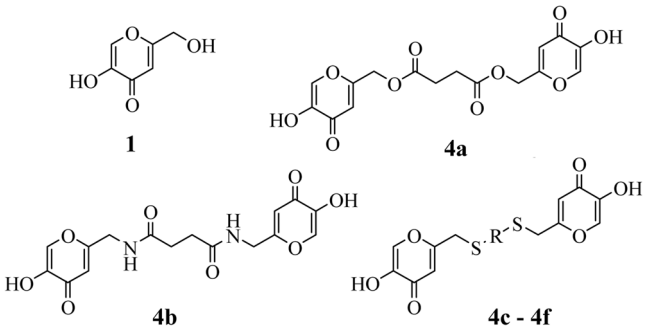
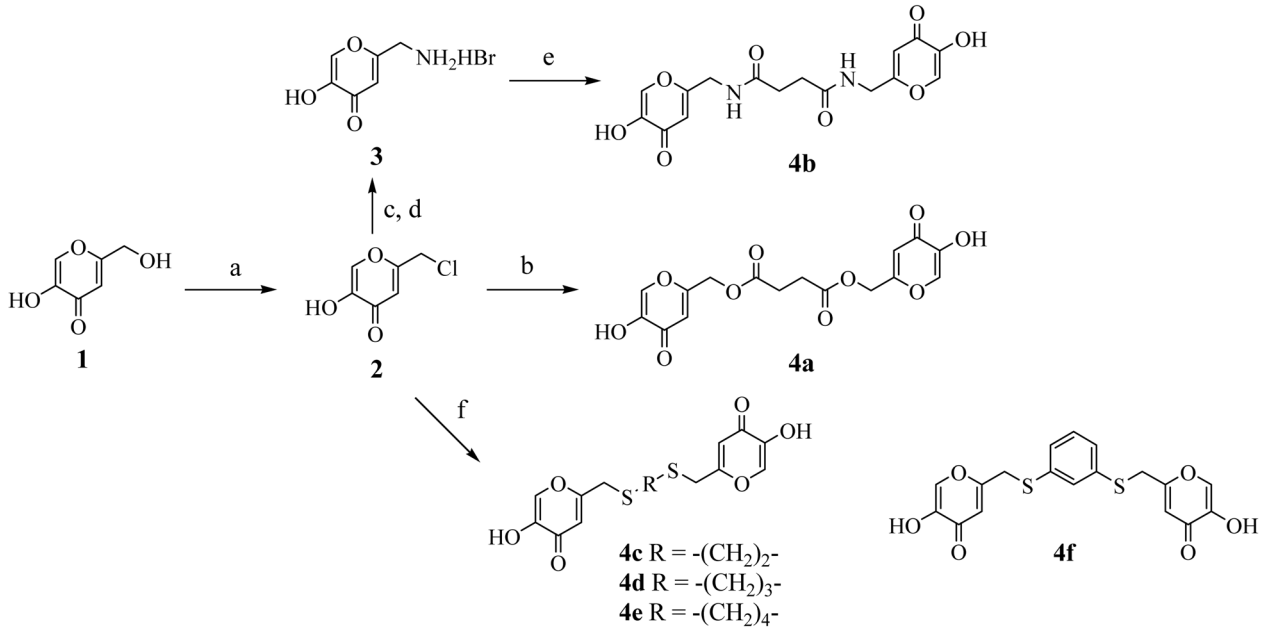


Figure 1. Structure of kojic acid **1** and the newly synthesized derivatives **4a-4f**.

Preparation of 4b. To a solution of kojyl amine HBr **3**[10] (6.7 g, 30 mmol) and TEA (7.0 g, 70 mmol) in THF (100 mL) under N_2 was added succinyl chloride (2.3 g, 15 mmol) dropwise. The reaction mixture was stirred for 1 h at room temperature and then THF was evaporated *in vacuo*. The residue was extracted with ethyl acetate (300 mL), washed with water. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate-hexane to give a **4b** (9.9 g) in 91% yields. R_f = 0.35 (SiO_2, 2:1 EtOAc/hexanes); ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.07 (bs, 2H), 8.45 (t, 2H, J = 5.4 Hz), 8.01 (s, 2H), 6.23 (s, 2H), 4.13 (d, 4H, J = 5.4 Hz), 2.43 (s, 4H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ173.99, 171.97, 165.51, 145.90, 139.60, 110.52, 39.87, 30.43. IR ν_{max} (KBr) 3259, 2924, 1649, 1520 cm^{-1}. FABMS: (m/e) 365 $[M+H]^+$.

Preparation of 4c. To a stirred solution of kojyl chloride **2** (4.8 g, 30 mmol) and TEA (4.0 g, 40 mmol) in THF (100 mL) under N_2 was added 1,2-ethanedithiol (1.4 g, 15 mmol). The reaction mixture was stirred for 10 h at room temperature and then THF was evaporated *in vacuo*. The residue was extracted with ethyl acetate (300 mL), washed with water. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate-hexane to give a **4c** (9.0 g) in 88% yields. R_f = 0.39 (SiO_2, 2:1 EtOAc/hexanes); ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.01 (bs, 2H), 7.97 (s, 2H), 6.33 (s, 2H), 3.62 (s, 4H), 2.68 (s, 4H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.37, 165.33, 146.29, 140.45, 112.87, 32.62, 31.69. IR ν_{max} (KBr) 3291, 2909, 1658, 1518 cm^{-1}. FABMS: (m/e) 343 $[M+H]^+$.

Compound 4d. R_f = 0.41 (SiO_2, 2:1 EtOAc/hexanes); ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.02 (bs, 2H), 7.98 (s, 2H), 6.32 (s, 2H), 3.61 (s, 4H), 2.50 (t, 4H, J = 7.2 Hz), 1.72 (m, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.36, 165.36, 146.29, 140.40, 112.86, 32.70, 30.53, 28.83. IR ν_{max} (KBr)



Scheme 1. Reaction conditions; (a) $SOCl_2$, DMF; (b) potassium salt of kojyl succinic acid, DMF; (c) NaN_3, DMF; (d) HBr, HOAc, phenol; (e) succinyl chloride, TEA, THF; (f) dithiols, TEA, THF.

3292, 2909, 1660, 1518 cm^{-1}. FABMS: (m/e) 357 $[M+H]^+$.

Compound 4e. R_f = 0.45 (SiO_2, 2:1 EtOAc/hexanes); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.05 (bs, 2H), 7.97 (s, 2H), 6.30 (s, 2H), 3.55 (s, 4H), 2.45 (m, 4H), 1.49 (m, 4H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 174.37, 165.48, 146.28, 140.39, 112.77, 32.79, 31.34, 28.21. IR ν_{max} (KBr) 3298, 2911, 1634, 1542 cm^{-1}. FABMS: (m/e) 371 $[M+H]^+$.

Compound 4f. R_f = 0.46 (SiO_2, 2:1 EtOAc/hexanes); ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.13 (bs, 2H), 8.02 (s, 2H), 7.41 (s, 1H), 7.24 (m,3H), 6.30 (s, 2H), 4.19 (s, 4H), ^{13}C-NMR (75 MHz, DMSO-d_6): δ 173.76, 163.76, 145.90, 140.04, 135.52, 129.92, 129.79, 127.93, 112.85, 34.23. IR ν_{max} (KBr) 3291, 2909, 1658, 1518 cm^{-1}. FABMS: (m/e) 391 $[M+H]^+$.

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[11] 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 mL 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).

Cell culture. Melan-a melanocytes are highly pigmented, immortalized normal murine melanocyte cell line derived from C57BL/6 mice. The melan-a melanocytes used in this study were obtained from Dr. Dorothy Bennett (St. George's Hospital, London, UK). Cells were grown and maintained at 37 °C in an atmosphere of 95% air, 5% CO_2 in RPMI-1640 (Bio Whittaker, Walkersville, MA) supplemented to a final concentration of 10% heat-inactivated fetal bovine serum, 5 units/mL penicillin, 5 μg/mL streptomycin and 200 nM phorbol 12-myristate 13-acetate. Cells were passaged every 3 days with a maximal passage number of 33. Confluent monolayers of melanocytes were harvested with a mixture of 0.05% trypsin, 0.53 mM EDTA (Gibco BRL, Grand Island, NY)

Measurements of melanin content and cell viability. Melanin content and cell number were measured in melan-a melanocytes. One hundred thousand cells were seeded into each well of 24 well plate and drugs were treated in triplicate. Medium was changed daily and after 4 days of culture, the cells were lysed with 1 mL of 1 N NaOH and pipetted repeatedly to homogenize. For analysis, 200 μL of each crude cell extracts were transferred into 96-well plates. Relative melanin content was measured at 400 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tex instruments). Cell viability was determined by the crystal violet assay. The culture medium was removed from the 24-well culture plates and replaced with 0.5 mL of 0.1% crystal violet in 10% ethanol per well. The plates were stained for 5 min at room temperature and rinsed with D.W four times. Crystal violet retained by adherent cell was extracted with 1 mL of 95% ethanol. Absorbance was determined at 540 nm using ELISA reader.

Results and Discussion

The synthesized kojic acid derivatives (**4a**-**4f**) and kojic acid **1** were then tested for their enzymatic inhibitory activities against tyrosinase. As shown in Table 1, compound **4a** containing succinic ester linkage between two kojic acid molecules showed mild inhibitory activity (IC_{50} = 21.46 μM). On the other hand, compound **4b** containing succinic amide linkage exhibited decreasing activity (IC_{50} = 112.38

μM). Inhibitory activities were dramatically enhanced by changing linkage with dithioether. Compound **4d** containing propane dithioether linkage showed most potent activity (IC_{50} = 1.97 μM). Its IC_{50} was about 1/25 compared to that of kojic acid (IC_{50} = 49.62 μM). The inhibitory effects of compound **4c** (IC_{50} = 2.65 μM) and compound **4e** (IC_{50} = 2.96 μM) were slightly lower than that of compound **4d**. However, compound **4f** containing 1,3-benzene dithioether linkage showed decreasing activity. Hence, these results indicate that thioether group and flexible structure are important factors for the inhibitory activity against tyrosinase. After testing tyrosinase inhibitory activity, we evaluated the inhibitory potency against the melanin formation. The synthesized derivatives were assayed for their cytotoxicities and inhibitory effects in a murine melanocytes cell line (Melan-a). In cell-based assay, alkane dithioether derivatives (**4c**-**4e**) were also more potent than other derivatives (**4a**, **4b** and **4f**). Among three derivatives, compound **4e** containing butane dithioether linkage showed stronger inhibitory activity (IC_{50} = 1.68 μM). Its activity was about 1000 times more potent than that of kojic acid (IC_{50} = 1.74 mM). Compound **4d** containing propane dithioether linkage, which exhibited the highest activity in tyrosinase inhibition, showed moderate activity (IC_{50} = 8.38 μM) in comparison with compound **4e**. In the case of compound **4c** possessing ethane dithioether linkage, lower activity (IC_{50} = 26.27 μM) was detected. These finding suggest that chain length of dithioether linkage was important for the inhibition of melanin synthesis. Considering enhancing inhibitory activities in tyrosinase enzyme assay and malan-a cell based assay, we propose that one kojic acid moiety in dithioether derivatives (**4c**-**4e**) block the active site of tyrosianse and the other kojic acid moiety can bind to another essential domain of tyrosinase to exhibit the enhanced inhibitory activity.

In case of cell based assay, physical properties of dithioether derivatives are important factor for the inhibition of melanin synthesis. To exhibit inhibitory activities, derivatives must penetrate into the cell membrane. Thus appropriate hydrophobic/hydrophilic balance of derivatives is important for the inhibition of melanin synthesis. The inhibitory effects were increased along with the increasing chain length of linkage in between two kojic acid. To analyze hydrophobic/hydrophilic balance of compounds (**4c**-**4e**) and kojic acid, we calculated lop P value (Table 2).

Table 1. Anti-melanogenic activities of kojic acid derivatives **4a**-**4f**

Samples	Tyrosinase IC_{50}	Melanin formation IC_{50}	% Survival of melan-a cell
Kojic acid	49.62 μM	1.74 mM	97.12 (3.0 mM)
Compound **4a**	21.46 μM	–	96.30 (30 μM)
Compound **4b**	112.38 μM	–	97.20 (30 μM)
Compound **4c**	2.65 μM	26.27 μM	94.50 (30 μM)
Compound **4d**	1.97 μM	8.38 μM	95.49 (30 μM)
Compound **4e**	2.96 μM	1.68 μM	96.29 (30 μM)
Compound **4f**	95.12 μM	–	98.20 (30 μM)

–; Not effective

Table 2. Calculation of Log P values

Samples	Log P^a
Kojic acid	–1.111
Compound **4c**	0.50
Compound **4d**	0.76
Compound **4e**	1.16

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

Kojic acid (Log *P* = –1.111) is hydrophilic compound due to the presence of two hydroxyl groups at 2 and 5 positions. One kojic acid moiety and thioether linkage at 2 position was introduced as a hydrophobic moiety and that caused enhancing inhibitory activity. Log P value data are correlated fairly well with inhibitory activities of **4c**-**4e**. Compound **4e**, which exhibited the highest activity in melan-a cell assay, is believed to be most adequate in cell penetration because it has good balance in hydrophobic/hydrophilic characters.

In conclusion, six kojic acid derivatives (**4a**-**4f**), which have two molecules of kojic acid connected by various linkage such as ester, amide, and thioether were synthesized and evaluated as potent inhibitors on tyrosinase activity and melanin formation in melan-a cells. Among derivatives, alkane dithioether derivatives (**4c**-**4e**) showed stronger activities as compared to other derivatives. The linkage of dithioether group and flexibility of linkage are very important for enhancing anti-melanogenic activity of kojic acid. In tyrosinase enzyme assay, compound **4d** possessing propane dithioether group was the most active compound. Its activity (IC_{50} = 1.97 μM) is about 25 times more potent than that of kojic acid (IC_{50} = 49.62 μM). However, malan-a cell based assay showed a little different results. Compound **4e** possessing butane dithioether group exhibited superior inhibitory activity of melanin synthesis (IC_{50} = 1.68 μM) at a nontoxic concentration compared to kojic acid (IC_{50} = 1.74 mM). Surprisingly, its IC_{50} was approximately 1/1000 compared to that of kojic acid.

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