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Solid-phase synthesis of kojic acid-tripeptides and their tyrosinase inhibitory activity, storage stability, and toxicity

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Abstract—A small library of kojic acid-tripeptides (Ko-$X_1X_2X_3$) was prepared by solid-phase parallel synthesis and assayed to evaluate their tyrosinase inhibitory activity. Most of the kojic acid-tripeptides showed better activities than kojic acid. Kojic acid-FWY was the best compound, and it exhibited 100-fold tyrosinase inhibitory activity compared with kojic acid. In addition, their storage stabilities were approximately 15 times higher and their toxicity was lower than that of kojic acid.

1. Introduction

The production of melanin by melanocytes within the skin or hair is called melanogenesis and tyrosinase (EC 1.14.18.1) is involved in this process. Tyrosinase catalyzes the oxidation of tyrosine to dihydroxyphenylalanine, which is subsequently oxidized to melanochrome.[1] Kojic acid is well known as one of the most popular tyrosinase inhibitors and it has been widely used as a whitening or anti-browning agent.[2]

However, the inhibitory activity of kojic acid is not sufficiently potent for it to be used. Moreover, kojic acid is too unstable to be stored for a long time and it is not safe enough to be utilized as an additive in cosmetics or foods. To overcome these drawbacks, amino acid derivatives of kojic acid were developed, which exhibited enhanced inhibitory activity relative to kojic acid, but their synthetic yield was very low.[3]

In this study, a library of kojic acid-tripeptides was prepared effectively by the solid-phase method and their tyrosinase inhibitory activities, storage stabilities, and toxicities were investigated.

2. Results and discussion

In order to connect the N-terminal of the peptides via a urethane bond, kojic acid needs to be converted to the active ester form. In an effort to accomplish this, the selective protection of the hydroxy group at position 5 was attempted, in order to prevent it reacting to yield undesired products, because the α-hydroxy ketone moiety is known to be essential for tyrosinase inhibition.[4] When acetic anhydride or 1-acetyltriazo-(4,5-*b*)pyridine[5] was employed as a selective acetylating agent, the desired acetyl protected kojic acid **2** was obtained in only 24% and 17% yields, respectively. Although the following activation step of **2** with carbonyl diimidazole (CDI) gave **3** in an acceptable yield (72%), the overall yields were unsatisfactory, due to the low yield of the previous steps. Nevertheless, we observed an interesting result in the activation step, in that **3** was clearly precipitated to a white powder in THF owing to the dramatic solubility change as **2** was converted to **3**. Considering this large difference in solubility between **2** and **3**, we attempted to activate the kojic acid using CDI without a prior protection step. As expected, the less hindered hydroxy group at position 7 reacted preferentially with CDI and an immediate precipitation took place to give the desired kojic acid derivative **4** in 70% yield (Scheme 1).

The tripeptides were assembled on a 2-chlorotrityl chloride (CTC) (0.9–1.1 mmol/g) resin using solid-phase Fmoc chemistry. *N*-Fmoc-amino acid was quantitatively

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Keywords: Kojic acid; Tyrosinase; Inhibitor; Peptide; Solid phase.

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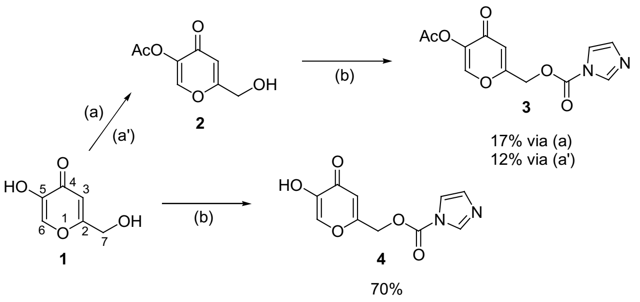
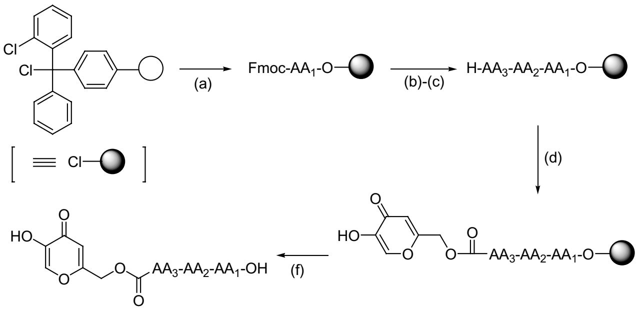


Scheme 1. Selective protection of hydroxyl group at position 5. Reagents and conditions: (a) acetic anhydride, TEA; (a′) 1-acetyltriazo-(4,5-*b*)pyridine, 1 N NaOH, THF; (b) carbonyl diimidazole, THF.



Scheme 2. Solid-phase kojic acid-tripeptides synthesis. Reagents and conditions: (a) Fmoc-AA_1-OH, DIPEA, NMP; (b) 20% piperidine in NMP; (c) Fmoc-AA-OH, BOP, 1-hydroxybenzotriazole (HOBt), NMP [repeat (b) and (c) two times]; (d) **4**; (f) reagent K.

introduced to the resin under diisopropylethylamine (DIPEA) in *N*-methylpyrrolidone (NMP) and then the general protocol of benzotriazole-1-yloxy-tris(dimethyl amino)-phosphonium hexafluorophosphate (BOP)-mediated coupling method afforded resin-bound tripeptides, which were further reacted with activated kojic acid, **4**. After final cleavage, kojic acid-tripeptides were obtained in overall yields of 49–95% (Scheme 2).

Tyrosinase inhibitory activities of the prepared kojic acid-tripeptides were evaluated by means of the mushroom tyrosinase inhibition assay method.[6] Thirty representative kojic acid-tripeptides were chosen and screened. Regardless of their sequences, all 30 of the tripeptide derivatives revealed the enhanced inhibitory activity compared with kojic acid. In particular, we found that the residue at the Ko+1 position played a key role. The phenylalanine residue with a hydrophobic side chain at the Ko+1 position exhibited better inhibitory activity than either lysine with a positively charged side chain or tyrosine with a polar side chain. This result suggests that hydrophobic side chains might contribute to their binding in the hydrophobic pocket near the active site of tyrosinase (Fig. 1).

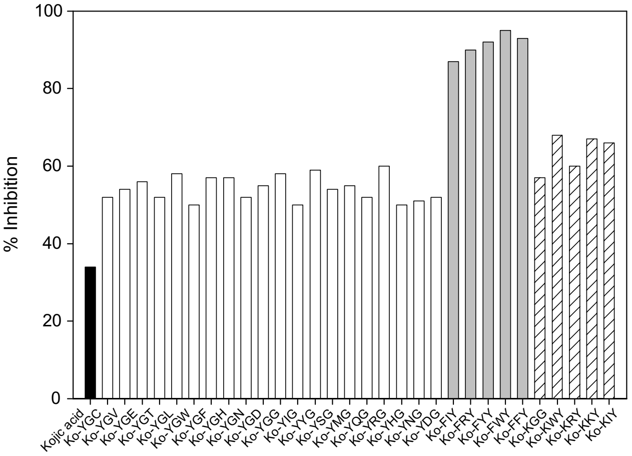


Figure 1. Tyrosinase inhibitory activities of kojic acid-tripeptides: inhibition degree was measured after treating 10 μM of each kojic acid-tripeptide solution, empty bars: Ko-YX_1X_2; solid bars: Ko-FX_1X_2; dashed bars: Ko-KX_1X_2.

Table 1. IC_{50} values of kojic acid and selected kojic acid-tripeptides

Inhibitor	IC_{50}, μM
Kojic acid	22.94
Ko-FWW	6.17
Ko-FWF	4.48
Ko-FWI	2.18
Ko-FWD	2.13
Ko-FYY	0.39
Ko-FFY	0.33
Ko-FWY	0.24
Ko-WWY	0.78

Based on the preliminary assay result, we focused our attention on the kojic acid-tripeptides with a phenylalanine residue at the Ko+1 position. The IC_{50} values of several Ko-FX_1X_2 were measured and they were found to exhibit 50–100 times higher inhibitory activity than kojic acid (Table 1). When the phenylalanine at the Ko+1 position was replaced with tryptophane, inhibitory activity was decreased. Interestingly, placing a tyrosine residue at the Ko+3 position induced an approximately 10-fold increase in the inhibitory activity.

Next, the storage stability of the kojic acid-tripeptides was examined. As candidates for this evaluation, five kojic acid-tripeptides (Ko-FRY, Ko-FFY, Ko-FYY, Ko-YWY) including the most active sequence (Ko-FWY) were selected and the change in their inhibitory activities was measured as a function of the storage time at ambient temperature. The results showed that the inhibitory activities of the kojic acid-tripeptides were maintained for more than 15 d. On the other hand, that of kojic acid was decreased rapidly after just one day (Fig. 2).

Finally, the cytotoxicities of the hydrophilic and hydrophobic kojic acid-tripeptides (Ko-KEY and Ko-FFY) were tested using the MTT assay.[7] From the assay results, we found that these kojic acid-tripeptides were less toxic than kojic acid and that they were safe even at high concentrations (up to 100 ppm) (Table 2).

In conclusion, a library of kojic acid-tripeptides was effectively prepared using the solid-phase method. The tyrosinase inhibitory activities of these kojic acid-tripeptides were higher than that of kojic acid. The inhibitory activity of Ko-FWY was the highest, with this

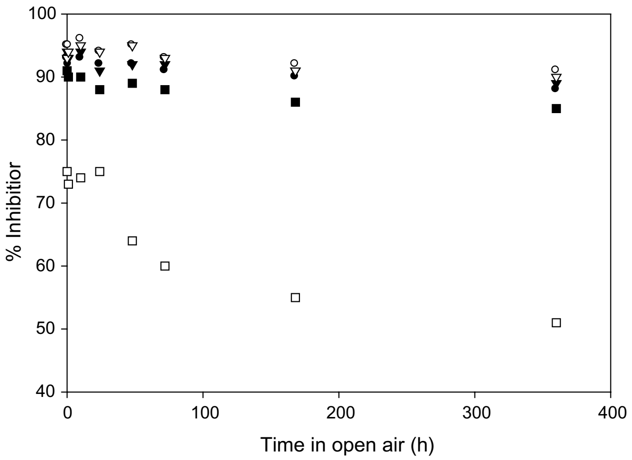


Figure 2. Storage stabilities (at rt) of kojic acid and kojic acid-tripeptides (□: kojic acid; ■: Ko-FRY; ▽: Ko-FFY; ▼: Ko-FYY; ○: Ko-FWY; ●: Ko-YWY).

Table 2. Cytotoxicity results of the selected kojic acid-tripeptides

	% Cell survive		
	1 ppm	10 ppm	100 ppm
Kojic acid	91.75	92.36	86.92
Ko-KEY	98.38	98.78	100.81
Ko-FFY	91.62	94.66	104.36
Ko-FWY	125.51	128.73	128.08
Ko-FYY	124.40	128.62	124.40

kojic acid-tripeptide being 90 times more active than kojic acid itself. Moreover, the kojic acid-tripeptides were more stable when stored under ambient conditions and were found to be atoxic. Further efforts to find better sequences of peptides by the pool-and-split method or a deconvolution approach are underway.

3. Experimental

3.1. Synthesis of 4

Kojic acid (**1**, 5 g, 35 mmol) and CDI (5.1 g, 0.9 equiv) were dissolved in THF (150 mL). After stirring for 24 h at room temperature, the resulting suspension was filtered, washed with THF, and dried under a high vacuum to give white solid in 70% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.33 (1H, s, –*OH*), 8.34 (1H, s, imidazole), 8.13 (1H, s, =*CH*CO), 7.67 (1H, s, imidazole), 7.10 (1H, s, imidazole), 6.67 (1H, s, =*CH*–O), 5.08 (2H, s, *CH*$_2$–OH).

3.2. Solid-phase synthesis of kojic acid-tripeptides

To a swollen 2-chlorotrityl chloride (CTC) resin (1.0 mmol of Cl/g, 1 g, 1.0 mmol) in NMP (10 mL) were added Fmoc-amino acid (1.5 equiv) and DIEA (1.5 equiv) and the reaction mixtures were stirred for 3 h. The resins were filtered, washed, and dried under high vacuum. The loading levels of the resulting resins were 0.84–0.95 mmol/g, which were confirmed by Fmoc titration. After deprotection of Fmoc with 20% piperidine in NMP for 30 min, the next amino acids were coupled to the resin by the general BOP-mediated solid-phase peptide synthesis protocol. Then, the activated kojic acid derivative, **4** (2 equiv) was added to the resin-bound tripeptide in NMP and the mixture was stirred for 3 h. Finally, the resin was treated with reagent K for 10 min.[8] The resin was filtered and washed. The collected filtrate was evaporated and precipitated with cold ether to give a white powder. The powder was filtered, washed with diethyl ether, and dried under a high vacuum to give the desired kojic acid-tripeptide.

3.3. Mushroom tyrosinase inhibition assay

Phosphate buffer (500 μL, pH 6.8, 0.1 M), 500 μL of tyrosine solution (0.3 mg/mL in water), 50 μL of tyrosinase solution (2 units/μL in phosphate buffer), and 400 μL of water were mixed in a micro-tube, and then 50 μL of the test substance were added. This solution (B) was stirred at 37 °C for 10 min, and then immediately cooled down using ice. After standing for 5 min on ice, the UV absorbance of this solution was measured at 490 nm. The same solution without test substance (A) was also prepared and the UV absorbance was measured at 490 nm. The % inhibition was calculated using the formula $[(A-B)/A]\times 100$. The IC$_{50}$ value was calculated by varying the concentration of the test substance.

3.4. MTT assay[9]

Melan-a cells were grown in 10 mL of RPMI 1640 medium supplemented with antibiotics, 10% fetal bovine serum (FBS), and 20 nM TPA. The cells were subcultured every 6 d. The cell suspension was poured into a 24-well plate (10^5 cells/mL) and the cells were allowed to completely adhere to the plate overnight. Then, 10 mL of each test sample and kojic acid as a reference dissolved in a mixture of solvents (propylene glycol–ethyl alcohol–H_2O = 50:30:20) were added to the plate, which was then incubated at 37 °C for 3 d in a CO_2 incubator with an atmosphere containing the same solution. The medium was renewed everyday. The percentage of viable cells was determined by staining the cell population with crystal violet. Crystal violet solution (200 μL, 0.1%) dissolved in 10% ethyl alcohol was added to the culture plates. After incubation for 5 min at room temperature, the plates were washed with PBS. After being shaken with 1 mL of ethyl alcohol for 10 min, the optical density of the resulting supernatant was measured at 590 nm using an ELISA reader (Molecular Devices 09090, USA).

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References and notes

1. Curto, E. V.; Kwong, C. *Biochem. Pharmacol.* **1999**, *57*, 663.
2. (a) Takahashi, T.; Asai, I. *Nippon Nogeik.* **1993**, *9*, 55; (b) Ohyama, Y.; Mishima, Y. *Frag. J.* **1990**, *6*, 53.
3. Kobayashi, Y.; Kayahara, H.; Tadasa, K. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1303.
4. Gastel, M.; Bubacco, L.; Canters, G. W. *FEBS Lett.* **2000**, *474*, 228.
5. Paradisi, M. P.; Torrini, I. *Tetrahedron Lett.* **1986**, *27*, 5029.
6. Andrawis, A.; Kahn, V. *Biochem. J.* **1986**, *235*, 91.
7. (a) Mukaida, N.; Kawai, T.; Takahashi, H. *Jpn. J. Clin. Pathol.* **1985**, *33*, 1279; (b) Levitz, S. M.; Diamond, R. D. *J. Infect. Dis.* **1985**, *152*, 938.
8. King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255–266.
9. Lee, S. H.; Lee, B. G.; Gao, J. J.; Kim, S. Y. *Biol. Pharm. Bull.* **2002**, *25*, 1045.