The Inhibitory Effect of New Hydroxamic Acid Derivatives on Melanogenesis

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The aim of present study was to examine the inhibitory effects of hydroxamic acid derivatives on the melanogenesis. We found that hydroxamic acid moiety was important for anti-melanogenic activity. Compounds **1a** and **1b** strongly inhibited melanin synthesis *via* deactivation of tyrosinase. Hydroxamic acid has metal ion chelating ability which is similar to that kojic acid, however, anti-tyrosinase mechanism of compounds **1a** and **1b** was different from that of kojic acid. They showed noncompetitive inhibition kinetics

Key Words : Hydroxamic acid, Derivative, Tyrosinase, Melanogenesis

Introduction

Skin hyperpigmentation can be dependent on either an increased number of melanocytes or activity of melanogenic enzymes, such as tyrosinase. Tyrosinase[1] is copper-containing enzyme that catalyzes the oxidation of tyrosine into dopa and subsequently dopaquinone. In the formation of melanin, three types of tyrosinase (met-, oxy-, and deoxy-tyrosinase) with different binuclear copper structures of the active site are involved.[2] On the basis of the consideration of copper structure in tyrosinase, many efforts have been focused on the regulation of tyrosinase activity using metal chelating compounds such as kojic acid,[3] catechol,[4] gentisic acid[5] and flavonol.[6] Hydroxamic acids[7] have been the source of much biochemical interest in recent years due to the fact that they show a wide range of biological activities. Much of their activities are due to their chelating properties with metal ions.[8] Although hydroxamic acids were well known as metal ion chelators, their efficacy data on tyrosinase inhibition and melanin biosynthesis were rare.[9] In this study, we synthesized new hydroxamic acid derivatives containing adamantane moiety and evaluated their inhibitory activities on melanogenesis (Fig. 1).

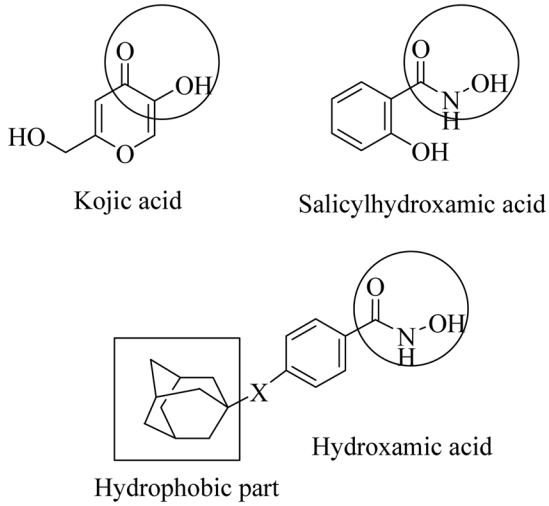
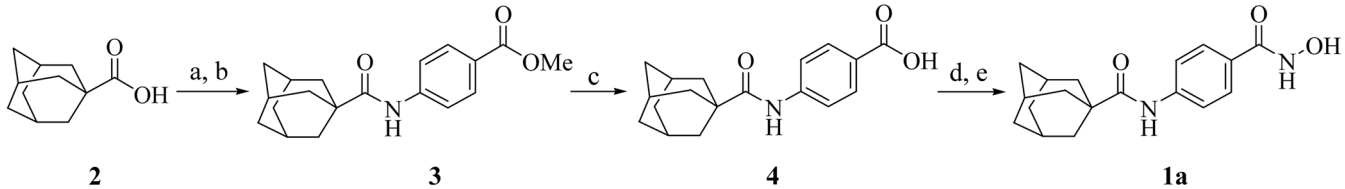


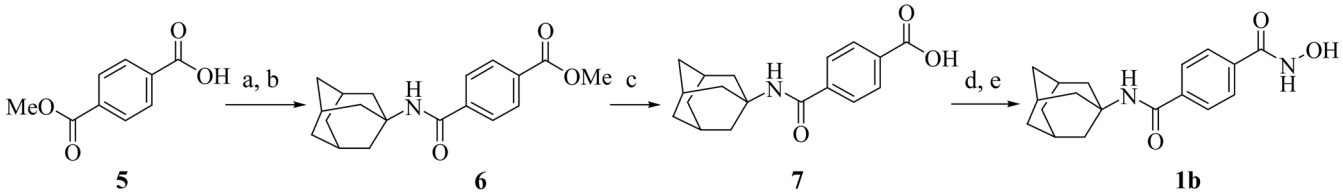
Figure 1. Structure of kojic acid and hydroxamic acid derivative **1a** and **1b**.

Results and Discussion

The synthetic pathways are shown in Scheme 1 and Scheme 2. 1-Adamantanecarboxylic acid (**2**) or monomethyl terephthalate (**5**) was refluxed in thionyl chloride to afford acid chlorides. These acid chlorides were reacted immediately with methyl 4-aminobenzoate and 1-adamantamine HCl in pyridine to produce corresponding amide derivatives (compounds **3** and **6**). The ester groups were hydrolyzed under standard condition (NaOH, ethanol) to produce corresponding acids (compounds **4** and **7**). The acids were reacted with ethylchloroformate and *N*-methyl morpholine in THF to convert the carboxylic acids to an anhydrides.[10] The anhydrides were reacted immediately with hydroxylamine to produce the corresponding hydroxamic acid derivatives (compound **1a** and **1b**).



Scheme 1. Reaction conditions; (a) $SOCl_2$, reflux; (b) methyl 4-aminobenzoate, pyridine; (c) NaOH, EtOH; (d) ethylchloroformate, *N*-methylmorpholine, THF; (e) hydroxylamine HCl, TEA, DMF.



Scheme 2. Reaction conditions; (a) $SOCl_2$, reflux; (b) 1-adamantamine HCl, pyridine; (c) NaOH, EtOH; (d) ethylchloroformate, *N*-methylmorpholine, THF; (e) hydroxylamine HCl, TEA, DMF

Table 1. Anti-melanogenic activities of hydroxamic acid derivatives

Compound	Melanocyte Pigmentation IC_{50} (μM)	Melanocyte Cytotoxicity IC_{50} (μM)
Compound **1a**	3.78	10.92
Compound **1b**	7.46	19.51
Compound **4**	> 50	> 50
Compound **7**	> 50	> 50
Salicylhydroxamic acid	> 50	> 50
Kojic acid	> 2 mM	> 2 mM

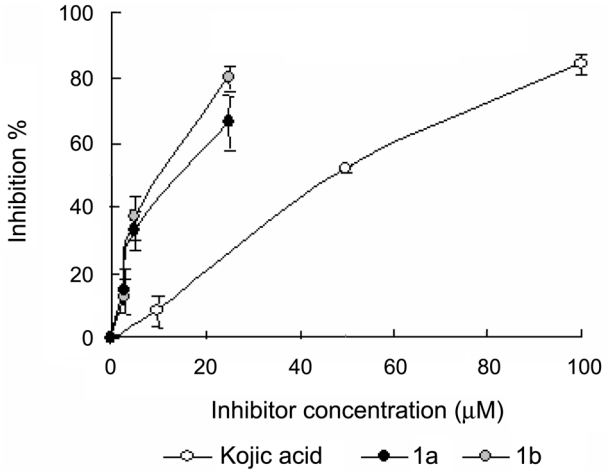


Figure 1. Dose-dependent inhibitory effects on mushroom tyrosinase by compound **1a**, **1b** and kojic acid. Effect on tyrosinase activity by samples as a function of concentration are represented as inhibition %, means ± S.E. of the three independent tests.

To investigate the anti-melanogenic properties in detail, hydroxamic acid derivatives (**1a** and **1b**) were assayed together with its synthetic precursors (compound **4** and compound **7**) and a standard tyrosinase inhibitor, salicylhydroxamic acid and kojic acid. Their depigmenting effects and cytotoxicities were determined in a murine melanocyte cell line (Melan-a). As shown in Table 1, compound **1a**, containing hydroxamic acid moiety, exhibited potent inhibitory activity (IC_{50} = 3.78 μM) with moderate cytotoxicity (IC_{50} = 10.92 μM). Compound **1b** showed similar behavior. The IC_{50} of pigmentation was about 7.46 μM. Compound **4** and **7**, precursors of hydroxamic acid derivatives, showed no inhibitory activities. These results suggest that hydroxamic acid moiety played an important role in anti-melanogenic activity. However, salicylhydroxamic acid,[9] known as a tyrosinase inhibitor in literature, exhibited no inhibitory activity in tested concentrations. Adamantane moiety of compound **1a** and **1b** is also important role for the inhibitory activity as well as hydroxamic acid. Recently, we evaluated compound **1b**,[12] as an antioxidant in lipid peroxidation both *in vitro* and *in vivo* studies. Adamantane group was adopted

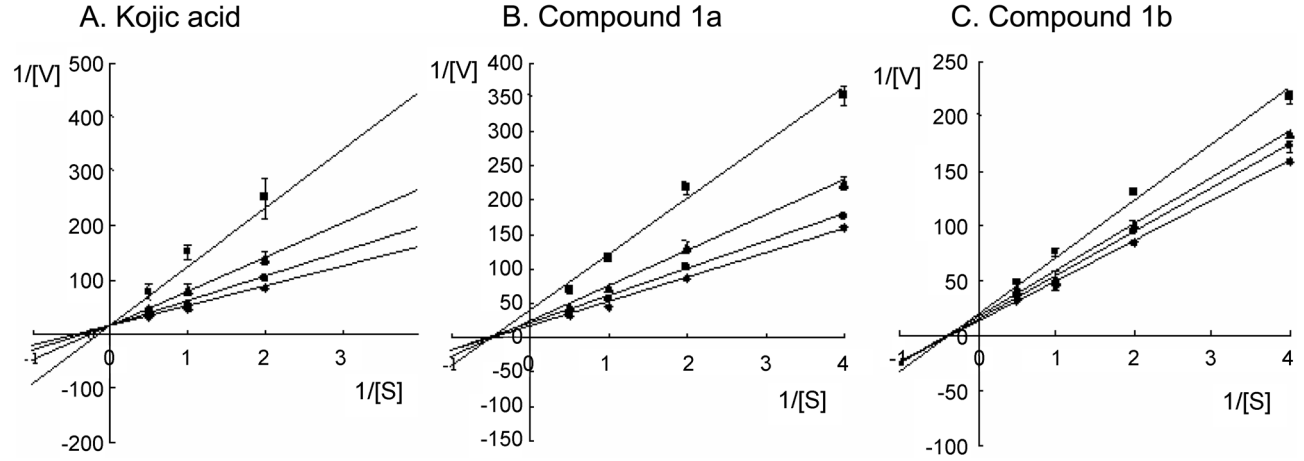


Figure 2. Lineweaver-Burk plot of mushroom tyrosinase on the presence of kojic acid, compound **1a** and **1b**. Data were obtained as mean value of 1/[V], inverse of the increase of optical density at 450 nm per min. (OD450/min), of three independent tests with different concentrations of L-tyrosine as a substrate. (A) with 100 μM (rectangle), 50 μM (triangle), 25 μM (circle), or no kojic acid (B) with 40 μM (rectangle), 20 μM (triangle), 10 μM (circle), or no compound **1a** (C) with 20 μM (rectangle), 10 μM (triangle), 5 μM (circle), or no compound **1b**.

as a hydrophobic moiety to optimize antioxidant activity of hydroxamic acid in animal test. In this cell based assay, compound **1a** and **1b** are also believed to be adequate in cell adhesion and permeation due to its balance in hydrophilic (hydroxamic acid moiety) and hydrophobic character (adamantane moiety). Kojic acid did not inhibit pigmentation at a concentration of up to 2 mM and showed no cytotoxicity at this concentration.

To clarify the influence of hydroxamic acid moiety on melanogenesis, we also measured tyrosinase activity after treatment with compound **1a** and **1b**. Tyrosinase is the key enzyme in melanin biosynthesis, and a hallmark of differenciation in melanocytes. Anti-tyrosinase activities of compound **1a** (IC_{50} = 12.04 μM) and **1b** (IC_{50} = 8.99 μM) were about 5 times higher than that of kojic acid (IC_{50} = 50.20 μM). These results indicate that compound **1a** and **1b** decreased melanin synthesis *via* deactivation of tyrosisnas in a dose-dependent manner (Fig. 1).

To determine inhibition type of hydroxamic acid derivatives on mushroom tyrosinase, the kinetic behavior was studied. A kinetic study of L-tyrosine oxidation catalyzed by mushroom tyrosinase was accomplished in the presence of compound **1a**, **1b** and kojic acid (Fig. 2). Michaelis-Menten constant (K_m value) of compound **1a** and **1b** were different from that of kojic acid. Through Lineweaver-Burk plot data, it was found that compound **1a** and **1b** were non-competitive inhibitor.[13] These behavior indicated that hydroxamic acid moiety did not block the active site itself but bound to an other essential domain of tyrosinase.

Conclusion

The inhibitory effects of hydroxamic acid derivatives on the melanogenesis have been studied. Results showed that hydroxamic acid derivatives strongly inhibited melanin synthesis *via* deactivation of tyrosinase. The presence of hydroxamic acid moiety was critical for anti-melanogenic activity. Although hydroxamic acid has metal ion chelating ability which is similar to that kojic acid, its anti-tyrosinase mechanism was different from that of kojic acid. Further studies on their more detail inhibition pathway in melanogenesis are underway.

Experimental Section

Preparation of Methyl 4-(Adamantanylcarbamoyl)-benzoate (2). 1-Adamantanecarboxylic acid **2** (9.0 g, 0.05 mol) was dissolved in 100 mL of $SOCl_2$ at 0 °C and refluxed for 1 h. The $SOCl_2$ was removed *in vacuo*, and crude acid chloride was dissolved in CH_2Cl_2 (50 mL). To a solution of methyl 4-aminobenzoate (7.5 g, 0.05 mol) in pyridine (100 mL) was added prepared acid chloride solution. The reaction mixture was stirred for 4 h at room temperature. The reaction mixture was concentrated *in vacuo* and the residue was extracted with ethyl acetate (250 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 dichloromethane-hexane to give a methyl 4-(adamantanylcarbamoyl)benzoate **2** (13.3 g) in 85% yields.

R_f = 0.68 (2:1 EtOAc/hexanes). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.93 (d, 2H, J = 8.1 Hz), 7.74 (d, 2H, J = 8.1 Hz), 7.49 (s, 1H), 3.85 (s, 3H), 2.05 (s, 9H), 1.59 (s, 3H). FABMS, *m/e* 314 $[M+H]^+$.

Preparation of 4-(Adamantanylcarbamoyl)benzoic Acid (3). Methyl 4-(adamantanylcarbamoyl)benzoate **2** (10.0 g, 0.03 mol) was dissolved in KOH (0.5 M, 300 mL) solution and heated to 50 °C. After obtaining clear solution, the clear solution was acidified with HCl (1 M) solution and resulting precipitate was gathered by filteration to give 4-(adamantanylcarbamoyl)benzoic acid **3** (8.6 g) in 90% yields.

R_f = 0.30 (2:1 EtOAc/hexanes). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.91 (d, 2H, J = 8.1 Hz), 7.76 (d, 2H, J = 8.1 Hz), 7.60 (s, 1H), 2.08 (s, 9H), 1.60 (s, 3H). FABMS, *m/e* 300 $[M+H]^+$.

Prepation of 4-(Adamantanecarboxamido)-*N*-hydroxybenzamide (1a). To a solution of 4-(adamantanylcarbamoyl)benzoic acid **3** (1g, 3.3 mmol) and *N*-methylmorpholine (400 mg, 3.9 mmol) in THF (10 mL) at 0 °C were added ethylchloroformate (423 mg, 3.9 mmol) dropwise and the mixture was stirred for 30 min. The solid was filtered off and the filtrate was added to the solution of hydroxylamine hydrochloride (229 mg, 3.3 mmol) and TEA (394 mg, 3.9 mmol) in DMF (10 mL) for 10 min. The reaction mixture was stirred for 30 min at 25 °C. DMF was evaporated *in vacuo*. The residue was extracted with ethyl acetate (50 mL), washed with water. The solvent was dried over $MgSO_4$ and evaporated to dryness. The crude product was purified by silica gel column chromatography to 4-(adamantanecarboxamido)-*N*-hydroxybenzamide **1a** (840 mg) in 81% yields.

R_f = 0.39 (2:1 EtOAc/hexanes). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 11.10 (s, 1H), 9.25 (s, 1H), 8.89 (s, 1H), 7.65 (m, 4H), 1.95 (s, 3H), 1.85 (s, 6H), 1.64 (s, 6H). IR ν_{max} (KBr) 3291, 2909, 1658, 1518 cm^{-1}. FABMS, *m/e* 315 $[M+H]^+$.

***N*^{1}-Adamantyl-*N*^{4}-hydroxyterephthalamide (1b).** R_f = 0.41 (2:1 EtOAc/hexanes). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 11.20 (s, 1H), 9.12 (s, 1H), 7.80 (m, 4H), 7.61 (s, 1H), 2.10 (s, 9H), 1.78 (s, 6H). IR ν_{max} (KBr) 3298, 2911, 1634, 1542 cm^{-1}. FABMS, *m/e* 315 $[M+H]^+$.

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 microliters 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 mL of 95% ethanol. Absorbance was determined at 540 nm using ELISA reader.

References

1. Penalver, M. J.; Hiner, A. N. P.; Rodriguez-Lopez, J. N.; Garcia-Canovas, F.; Tudela, J. *Biochim. Biophys. Acta* **2002**, *1597*, 140.
2. Fenoll, L. G.; Rodriguez-Lopez, J. N.; Garcia-Sevilla, F.; Garcia-Ruiz, P. A.; Varon, R.; Garcia-Canovas, F.; Tudela, J. *Biochim. Biophys. Acta* **2001**, *1548*, 1.
3. Ohyama, Y.; Mishima, Y. *Fragrance J*. **1990**, *6*, 53.
4. (a) Cho, S. J.; Roh, J. S.; Sun, W. S.; Kim, S. H.; Park, K. D. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2682. (b) Khatib, S.; Nerya, O.; Musa, R.; Shmuel, M.; Tamir, S.; Vaya, J. *Bioorg. Med. Chem.* **2005**, *13*, 433.
5. Curto, E. V.; Kwong, C.; Hermerdorfer, H.; Glatt, H.; Santis, C.; Virador, V.; Hearing, V. J.; Dooley, T. P. *Biochem. Pharmacol.* **1999**, *57*, 663.
6. (a) Kubo, I.; Kinst-Hori, I.; Chaudhuri, S. K.; Kubo, Y.; Sanchez, Y.; Ogura, T. *Bioorg. Med. Chem.* **2000**, *8*, 1749. (b) Makris, D. P.; Rossiter, J. T. *Food Chemistry* **2002**, *77*, 177.
7. (a) Taira, J.; Miyagi, C.; Aniya, Y. *Biochem. Pharmacol.* **2002**, *63*, 1019. (b) Pirrung, M. C.; Tumey, L. N.; McClerren, A. L.; Raetz, C. R. H. *J. Am. Chem. Soc.* **2003**, *125*, 1575. (c) Denicola, A.; Souza, J. M.; Gatti, R. M.; Augusto, O.; Radi, R. *Free Radic. Biol. Med*. **1995**, *19*, 11. (d) Vanjari, H.; Pande, R. *J. Pharm. Biomed. Anal.* **2003**, *33*, 783.
8. Farkas, E.; Enyedy, E. A.; Micera, G.; Garribba, E. *Polyhedron* **2000**, *19*, 1727.
9. Rich, P. R.; Wiegand, N. K.; Blum, H.; Moore, A. L.; Bonner, W. D. *Biochim. Biophys. Acta* **1978**, *525*, 325.
10. Reddy, A. S.; Kumar, M. S.; Reddy, G. R. *Tetrahedron Lett*. **2000**, *41*, 6285.
11. Pomerantz, S. H. *J. Biol. Chem*. **1963**, *238*, 2351.
12. Rho, H. S.; Baek, H. S.; Kim, D. H.; Chang, I. S. *Bull. Korean Chem. Soc*. **2006**, *27*, 584.
13. (a) Shi, Y.; Chen, Q.-X.; Wang, Q.; Song, K.-K.; Qiu, L. *Food Chemistry* **2005**, *92*, 707. (b) Shin, N.-H.; Ryu, S. Y.; Choi, E. J.; Kang, S.-H.; Chang, I.-M.; Min, K. R.; Kim, Y. *Biochem. Biophys. Res. Commun.* **1998**, *243*, 801.