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Design and Synthesis of Hydroxypyridinone-L-phenylalanine Conjugates as Potential Tyrosinase Inhibitors

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ABSTRACT: A range of hydroxypyridinone-L-phenylalanine conjugates were synthesized starting from kojic acid. Their tyrosinase activity was determined, and it was found that one of the compounds ((*S*)-(5-(benzyloxy)-1-octyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-amino-3-phenylpropanoate, **5e**) showed potent inhibitory effect against mushroom tyrosinase, the IC_{50} values for monophenolase and diphenolase activities being 12.6 and 4.0 μM, respectively. It was also demonstrated that these conjugates are mixed-type inhibitors, suggesting they could bind to both the free enzyme and the enzyme−substrate complexes. MTT assay indicated that **5e** was nontoxic to three cell lines. This compound may find applications in food preservation and cosmetics.

KEYWORDS: *hydroxypyridinone, kojic acid, tyrosinase inhibitor*

INTRODUCTION

The production of melanin by melanocytes, melanogenesis, is initiated by tyrosinase upon exposure of the skin to UV radiation. Tyrosinase (EC 1.14.18.1), a multifunctional copper-containing enzyme, is widely distributed in plants and animals,[1] and plays an important role in the pathway of melanin biosynthesis from L-tyrosine. It is well-known that tyrosinase can catalyze the first and rate-limiting step of melanin formation, the hydroxylation of L-tyrosine to L-3-(3,4-dihydroxyphenyl)-alanine (L-DOPA) (monophenolase activity) and also the subsequent oxidation of DOPA to dopaquinone (diphenolase activity). Dopaquinone is highly reactive and can polymerize spontaneously to form melanin in a series of reaction pathways.[2] The enzymatic oxidation of L-tyrosine into melanin is of considerable importance in the coloring of skin, hair, and eyes and in food browning.[3,4] It has also been suggested that tyrosinase contributes to the neurodegeneration associated with Parkinson's disease.[5,6] Thus, the development of novel tyrosinase inhibitors is of interest to agricultural, cosmetic, and medical fields.[7−10] A large number of naturally occurring and synthetic compounds that are tyrosinase inhibitors have been reported.[11,12] However, only a few of them are sufficiently potent for practical use and comply with general safety regulations. kojic acid (**1**), 5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one, which is isolated from metabolic products of many species of *Aspergillus* and *Penicillium* molds, possesses appreciable inhibitory activity against tyrosinase and also possesses free radical scavenging activity.[13,14] Kojic acid has been used as an ingredient in cosmetics and as an antibrowning agent in foods that rapidly change color.[15,16] Thus, modification of kojic acid provides a potential route for superior tyrosinase inhibitors.[17−20] As kojic acid can inhibit tyrosinase by chelating copper ion in the active site of tyrosinase due to the ketone group at position 4 and the hydroxyl group at position 5, it is assumed that an increase of affinity for copper may result in a stronger tyrosinase inhibition. As 3-hydroxypyridin-4-ones are predicted to possess a higher copper affinity than that of the corresponding hydroxypyranones, we have synthesized a range of 3-hydroxypyridin-4-one derivatives (**5**) with a hydrophobic alkyl group at the N-1 position, and their tyrosinase inhibitory activities have been investigated.

MATERIALS AND METHODS

Instruments. ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer or a Bruker Avance 500 spectrometer (Bruker Corp., Germany) with tetramethylsilane as an internal standard. Electrospray ionization (ESI) mass spectra were obtained by infusing samples into an LCQ Deca XP ion-trap instrument (ThermoFinnigan, San Jose, CA). High-resolution mass spectra (HRMS) were obtained on a QTOFMicro (Waters, U.S.) by direct infusing samples into the ESI source.

General Synthesis. All chemicals were of AR grade and used without any further purification.

Synthesis of 5-(Benzyloxy)-2-(hydroxymethyl)-4H-pyran-4-one (2). To a solution of kojic acid (71 g, 0.5 mol) in methanol (150 mL) was added NaOH solution (22 g in 50 mL of H_2O). The mixture was heated to reflux, BnCl (75.9g, 0.6 mol) was added dropwise over 1.5 h, and the reflux was continued for 6 h. The reaction mixture was cooled to room temperature, filtered to remove the inorganic salt, and the filtrate was cooled. White solid was precipitated and filtered. The collected

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product was washed with cold methanol and obtained as a white solid **2** (83 g, 72% yield).

*General Procedure for the Synthesis of **3**.* A mixture of **2** (5 mmol), NaOH (1.0 g), and amine RNH_2 (5.5 mmol) in $MeOH/H_2O$ (20 mL/10 mL) was refluxed for 2−3 h. The reaction was monitored by TLC. After completion of the reaction, the mixture was concentrated to about half volume, and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layer was washed with brine, and dried over anhydrous Na_2SO_4. After removal of the solvent, the crude product **3** was obtained, which was used in the next reaction without further purification.

*5-(Benzyloxy)-2-(hydroxymethyl)-1-methylpyridin-4(1H)-one (**3a**).* ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.58 (s, 3H, CH_3), 4.36 (s, 2H, CH_2), 4.98 (s, 2H, CH_2), 6.22 (s, 1H, C6−H in pyridinone ring), 7.31−7.41 (m, 5H, Ph), 7.55 (s, 1H, C3−H in pyridinone ring). ESI−MS: m/z 246 (MH^+).

*5-(Benzyloxy)-2-(hydroxymethyl)-1-ethylpyridin-4(1H)-one (**3b**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.29 (t, J = 7.2 Hz, 3H, CH_3), 3.96 (q, J = 7.2 Hz, 2H, CH_2), 4.44 (s, 2H, CH_2), 5.11 (s, 2H, CH_2), 6.32 (s, 1H, C6−H in pyridinone ring), 6.94 (s, 1H, C3−H in pyridinone ring), 7.27−7.38 (m, 5H, Ph). ESI−MS: m/z 260 (MH^+).

*5-(Benzyloxy)-2-(hydroxymethyl)-1-butylpyridin-4(1H)-one (**3c**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.85 (t, J = 7.2 Hz, 3H, CH_3), 1.14 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 3.92 (q, J = 7.2 Hz, 2H, CH_2), 4.43 (s, 2H, CH_2), 5.09 (s, 2H, CH_2), 6.32 (s, 1H, C6−H in pyridinone ring), 6.92 (s, 1H, C3−H in pyridinone ring), 7.27−7.38 (m, 5H, Ph). ESI−MS: m/z 288 (MH^+).

*5-(Benzyloxy)-2-(hydroxymethyl)-1-hexylpyridin-4(1H)-one (**3d**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, J = 6.8 Hz, 3H, CH_3), 1.21 (m, 6H, CH_2), 1.58 (m, 2H, CH_2), 3.87 (q, J = 7.6 Hz, 2H, CH_2), 4.43 (s, 2H, CH_2), 5.10 (s, 2H, CH_2), 6.34 (s, 1H, C6−H in pyridinone ring), 6.91 (s, 1H, C3−H in pyridinone ring), 7.27−7.39 (m, 5H, Ph). ESI−MS: m/z 316 (MH^+).

*5-(Benzyloxy)-2-(hydroxymethyl)-1-octylpyridin-4(1H)-one (**3e**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, J = 7.2 Hz, 3H, CH_3), 1.15 (m, 2H, CH_2), 1.23 (m, 8H, CH_2), 1.51 (m, 2H, CH_2), 3.88 (q, J = 7.2 Hz, 2H, CH_2), 4.42 (s, 2H, CH_2), 5.10 (s, 2H, CH_2), 6.31 (s, 1H, C6−H in pyridinone ring), 6.89 (s, 1H, C3−H in pyridinone ring), 7.27−7.38 (m, 5H, Ph). ESI−MS: m/z 344 (MH^+).

*5-(Benzyloxy)-2-(hydroxymethyl)-1-decylpyridin-4(1H)-one (**3f**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.85 (t, J = 6.8 Hz, 3H, CH_3), 1.22 (m, 14H, CH_2), 1.60 (m, 2H, CH_2), 3.86 (q, J = 7.2 Hz, 2H, CH_2), 4.42 (s, 2H, CH_2), 5.09 (s, 2H, CH_2), 6.33 (s, 1H, C6−H in pyridinone ring), 6.92 (s, 1H, C3−H in pyridinone ring), 7.28−7.39 (m, 5H, Ph). ESI−MS: m/z 372 (MH^+).

*General Procedure for the Synthesis of **4**.* A mixture of compound **3** (5 mmol), *N*-Cbz-L-phenylalanine (5.5 mmol), EDC·HCl (5.5 mmol), and DMAP (0.2 g) in DMF (20 mL) was stirred at room temperature. After removal of the solvent, the residue was dissolved in dichloromethane (100 mL), washed with brine (2 × 50 mL), and dried over anhydrous Na_2SO_4. After filtration, the filtrate was concentrated, and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH, 10:1), giving the product **4** as a pale yellow powder.

*(S)-(5-(Benzyloxy)-1-methyl-4-oxo-1,4-dihydropyridin-2-yl)-methyl 2-(Benzyloxycarbonyl)-3-phenylpropanoate (**4a**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.06 (m, 2H, CH_2), 3.26 (s, 3H, CH_3), 4.61 (q, J = 7.2 Hz, 1H, CH), 4.87 (m, 2H, CH_2), 5.07 (s, 2H, CH_2), 5.18 (s, 2H, CH_2), 6.48 (s, 1H, C6−H in pyridinone ring), 6.86 (s, 1H, C3−H in pyridinone ring), 7.04 (m, 2H, Ph), 7.16 (m, 2H, Ph), 7.29−7.42 (m, 12H, 11 from Ph, 1 from NH). ESI−MS: m/z 527 (MH^+).

*(S)-(5-(Benzyloxy)-1-ethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-(Benzyloxycarbonyl)-3-phenylpropanoate (**4b**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17 (t, J = 7.2 Hz, 3H, CH_3), 3.07 (m, 2H, CH_2), 3.55 (m, 2H, CH_2), 4.62 (m, 1H, CH), 4.88 (m, 2H, CH_2), 5.07 (s, 2H, CH_2), 5.19 (s, 2H, CH_2), 6.46 (s, 1H, C6−H in pyridinone ring), 6.87 (s, 1H, C3−H in pyridinone ring), 7.03 (m, 2H, Ph), 7.18 (m, 2H, Ph), 7.29−7.41 (m, 12H, 11 from Ph, 1 from NH). ESI−MS: m/z 541 (MH^+).

*(S)-(5-(Benzyloxy)-1-butyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-(Benzyloxycarbonyl)-3-phenylpropanoate (**4c**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.85 (t, J = 7.2 Hz, 3H, CH_3), 1.14 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 3.06 (d, J = 6.4 Hz, 2H, CH_2), 3.50 (m, 2H, CH_2), 4.60 (q, J = 7.2 Hz, 1H, CH), 4.88 (m, 2H, CH_2), 5.06 (s, 2H, CH_2), 5.19 (s, 2H, CH_2), 6.44 (s, 1H, C6−H in pyridinone ring), 6.84 (s, 1H, C3−H in pyridinone ring), 7.02 (m, 2H, Ph), 7.18 (m, 2H, Ph), 7.27−7.39 (m, 12H, 11 from Ph, 1 from NH). ESI−MS: m/z 569 (MH^+).

*(S)-(5-(Benzyloxy)-1-hexyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-(Benzyloxycarbonyl)-3-phenylpropanoate (**4d**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (t, J = 7.2 Hz, 3H, CH_3), 1.13 (m, 2H, CH_2), 1.25 (m, 4H, CH_2), 1.43 (m, 2H, CH_2), 2.96 (d, J = 6.4 Hz, 2H, CH_2), 3.49 (m, 2H, CH_2), 4.55 (q, J = 6.8 Hz, 1H, CH), 4.86 (m, 2H, CH_2), 5.04 (s, 2H, CH_2), 5.19 (s, 2H, CH_2), 6.45 (s, 1H, C6−H in pyridinone), 6.85 (s, 1H, C3−H in pyridinone ring), 7.01 (m, 2H, Ph), 7.18 (m, 2H, Ph), 7.27−7.39 (m, 12H, 11 from Ph, 1 from NH). ESI−MS: m/z 597 (MH^+).

*(S)-(5-(Benzyloxy)-1-octyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-(Benzyloxycarbonyl)-3-phenylpropanoate (**4e**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, J = 7.2 Hz, 3H, CH_3), 1.13 (m, 2H, CH_2), 1.22 (m, 8H, CH_2), 1.49 (m, 2H, CH_2), 3.06 (d, J = 6.4 Hz, 2H, CH_2), 3.51 (m, 2H, CH_2), 4.61 (q, J = 6.8 Hz, 1H, CH), 4.88 (m, 2H, CH_2), 5.07 (s, 2H, CH_2), 5.19 (s, 2H, CH_2), 6.46 (s, 1H, C6−H in pyridinone ring), 6.86 (s, 1H, C3−H in pyridinone ring), 7.03 (m, 2H, Ph), 7.18 (m, 2H, Ph), 7.28−7.40 (m, 12H, 11 from Ph, 1 from NH). ESI−MS: m/z 625 (MH^+).

*(S)-(5-(Benzyloxy)-1-decyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-(Benzyloxycarbonyl)-3-phenylpropanoate (**4f**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.84 (t, J = 6.8 Hz, 3H, CH_3), 1.21 (m, 14H, CH_2), 1.61 (m, 2H, CH_2), 2.91 and 3.04 (m, 2H, CH_2), 3.76 (m, 2H, CH_2), 4.32 (m, 1H, CH), 4.93−5.08 (m, 6H, CH_2), 6.27 (s, 1H, C6−H in pyridinone ring), 7.19−7.41 (m, 15H, Ph), 7.59 (s, 1H, C3−H in pyridinone ring), 7.93 (d, J = 8.0 Hz, 1H, NH). ESI−MS: m/z 653 (MH^+).

*General Procedure for the Synthesis of **5**.* To a suspension of **4** (2 mmol) and benzyl chloride (5 mmol) in MeOH/EtOAc (15 mL/15 mL) was added 5% Pd/C (10% weight of **4**). Hydrogenation was carried out at 30 psi H_2 for 5−6 h at room temperature. After filtration to remove the catalyst, the filtrate was concentrated to dryness. The residue was redissolved in a small amount of methanol, and precipitated by the addition of diethyl ether. After removal of the supernatant, the residue was dried at 40 °C under vacuum, providing hydrochlorides of **5** as pale brown solids.

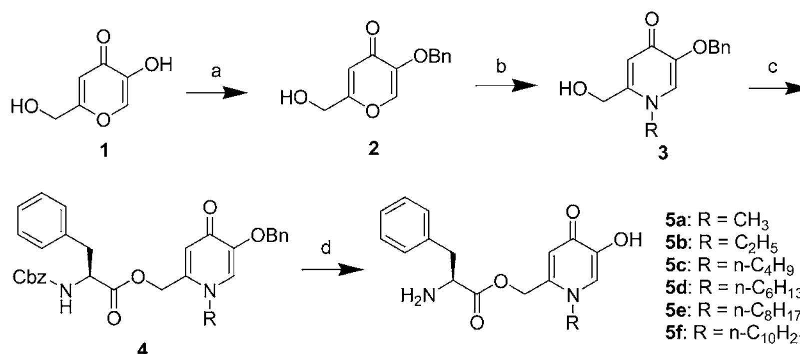
*Hydrochloride Salt of (S)-(5-(Benzyloxy)-1-methyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-Amino-3-phenylpropanoate (**5a**).* ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.25 (m, 2H, CH_2), 3.87 (t, J = 7.2 Hz, 3H, CH_3), 4.12 (m, 1H, CH), 5.29 (m, 2H, CH_2), 7.21 (m, 5H, Ph), 7.40 (s, 1H, C6−H in pyridinone ring), 8.39 (s, 1H, C3−H in pyridinone ring), 8.85 (br, 3H, NH_3^+). ESI−MS: m/z 303 (MH^+). HR−MS: m/z calcd for $C_{16}H_{19}N_2O_4$ (MH^+) 303.1345, found 303.1341.

*Hydrochloride Salt of (S)-(5-(Benzyloxy)-1-ethyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-Amino-3-phenylpropanoate (**5b**).* ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.33 (t, J = 7.2 Hz, 3H, CH_3), 3.11 and 3.25 (m, 2H, CH_2), 4.16 (m, 2H, CH_2), 4.32 (m, 1H, CH), 5.31 (m, 2H, CH_2), 7.21 (m, 5H, Ph), 7.39 (s, 1H, C6−H in pyridinone ring), 8.37 (s, 1H, C3−H in pyridinone ring), 8.87 (br, 3H, NH_3^+). ESI−MS: m/z 317 (MH^+). HR-MS: m/z calcd for $C_{17}H_{21}N_2O_4$ (MH^+) 317.1501, found 317.1496.

*Hydrochloride Salt of (S)-(5-(Benzyloxy)-1-butyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-Amino-3-phenylpropanoate (**5c**).* ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.89 (t, J = 7.2 Hz, 3H, CH_3), 1.29 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 3.10 and 3.24 (m, 2H, CH_2), 4.14 (m, 2H, CH_2), 4.32 (m, 1H, CH), 5.31 (s, 2H, CH_2), 7.21 (m, 5H, Ph), 7.38 (s, 1H, C6−H in pyridinone ring), 8.35 (s, 1H, C3−H in pyridinone ring), 8.86 (br, 3H, NH_3^+). ESI−MS: m/z 345 (MH^+). HR−MS: m/z calcd for $C_{19}H_{25}N_2O_4$ (MH^+) 345.1814, found 345.1805.

*Hydrochloride Salt of (S)-(5-(Benzyloxy)-1-hexyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-Amino-3-phenylpropanoate (**5d**).* ^{1}H NMR (500 MHz, MeOD): δ 0.91 (t, J = 7.0 Hz, 3H, CH_3), 1.35 (m, 4H, CH_2), 1.41 (m, 2H, CH_2), 1.87 (m, 2H, CH_2), 3.21 and 3.30 (m, 2H, CH_2), 4.23 (m, 1H, CH), 4.27 (t, J = 7.0 Hz, 2H, CH_2), 4.75 (s, 2H, CH_2), 7.31 (m, 6H, Ph and C6−H in pyridinone ring), 8.12

Scheme 1[a]



[a]Reagents and conditions: (a) BnCl, MeOH/H_2O, 70 °C, 6 h; (b) RNH_2, EtOH/H_2O, NaOH, reflux, 3 h, 72−85% yield; (c) EDC, DMAP; (d) H_2 (30 psi), Pd/C, EtOAc/MeOH (1:1), room temperature, 5 h, 88−93% yield.

(s, 1H, C3−H in pyridinone ring). ESI−MS: *m*/*z* 373 (MH^+). HR−MS: *m*/*z* calcd for $C_{21}H_{29}N_2O_4$ (MH^+) 373.2127, found 373.2132.

*Hydrochloride Salt of (S)-(5-(Benzyloxy)-1-octyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-Amino-3-phenylpropanoate (**5e**).* 88% yield. ^{1}H NMR (500 MHz, MeOD): δ 0.91 (t, *J* = 7.0 Hz, 3H, CH_3), 1.32 (m, 10H, CH_2), 1.86 (m, 2H, CH_2), 3.2 and 3.33 (m, 2H, CH_2), 4.25−4.32 (m, 3H, CH and CH_2), 4.78 (s, 2H, CH_2), 7.33 (m, 6H, Ph and C6−H in pyridinone ring), 8.18 (s, 1H, C3−H in pyridinone ring). ESI−MS: *m*/*z* 401 (MH^+). HR−MS: *m*/*z* calcd for $C_{23}H_{33}N_2O_4$ (MH^+) 401.2440, found 401.2447.

*Hydrochloride Salt of (S)-(5-(Benzyloxy)-1-decyl-4-oxo-1,4-dihydropyridin-2-yl)methyl 2-Amino-3-phenylpropanoate (**5f**).* ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.86 (t, *J* = 6.8 Hz, 3H, CH_3), 1.24 (m, 14H, CH_2), 1.70 (m, 2H, CH_2), 3.12 and 3.25 (m, 2H, CH_2), 4.16 (m, 2H, CH_2), 4.31 (m, 1H, CH), 5.33 (s, 2H, CH_2), 7.21 (m, 5H, Ph), 7.47 (s, 1H, C6−H in pyridinone ring), 8.42 (s, 1H, C3−H in pyridinone ring), 8.89 (br, 3H, NH_3^+). ESI−MS: *m*/*z* 429 (MH^+). HR−MS: *m*/*z* calcd for $C_{25}H_{37}N_2O_4$ (MH^+) 429.2753, found 429.2762.

Tyrosinase Inhibition Assay. Mushroom tyrosinase (lyophilized powder, ≥1000 unit/mg solid) was purchased from Sigma. One unit = ΔA_{280} of 0.001 per min at pH 6.5 at 25 °C in 3 mL of reaction mixture containing L-tyrosine.

Prepration of Tyrosinase Solution. Ten milligrams of mushroom tyrosinase was dissolved in distilled water and diluted to a final concentration of 100 unit/mL.

Inhibitory Effect of Hydroxypyridinone-L-phenylalanine Conjugates on the Tyrosinase Activity. In this investigation, L-tyrosine was used as the substrate for the monophenolase activity assay, and L-DOPA was used as the substrate for the diphenolase activity assay. The reaction system (3 mL) containing different concentrations of 0.1 mL inhibitors was first mixed with 1 mL of substrate solution containing 1.8 mL of 50 mM phosphate buffer (pH 6.8), then a portion of 0.1 mL of enzyme solution was added to this blend, and the reaction was carried out at 30 °C for 10 min. Absorption and kinetic measurements were carried out on a UV-2100 spectrophotometer at 475 nm. Controls, without inhibitors, were routinely carried out. The inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity (IC_{50}).

Determination of the Inhibition Type and Inhibition Constant. The inhibition type was assayed by the Lineweaver−Burk plot based on the results of inhibitory effect on the diphenolase activity of tyrosinase, and the inhibition constant was determined by the secondary plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor, as described by Chen et al.[21]

pK_a and Copper(II) Stability Constants' Determination. The automatic titration system used in this study was comprised of an autoburet (Metrohm Dosimat 765 L mL syringe), Mettler Toledo MP230 pH meter with Metrohm pH electrode (6.0133.100), and a reference electrode (6.0733.100). 0.1 M KCl electrolyte solution was used to maintain the ionic strength. The temperature of the test solutions was maintained in a thermostatic jacketed titration vessel at 25 ± 0.1 °C by using a Techne TE-8J temperature controller. The solution under investigation was stirred vigorously during the experiment. A Gilson Mini-plus#3 pump with speed capability (20 mL/min) was used to circulate the test solution through a Hellem quartz flow cuvette. For the stability constant determinations, a 50 mm path length cuvette was used, and for pK_a determinations, a cuvette path length of 10 mm was used. The flow cuvette was mounted on an HP 8453 UV−visible spectrophotometer. All instruments were interfaced to a computer and controlled by a Visual Basic program. Automatic titration and spectral scans adopted the following strategy: the pH of a solution was increased by 0.1 pH unit by the addition of KOH from the autoburet; when pH readings varied by <0.001 pH unit over a 3 s period, an incubation period was activated. For pK_a determinations, a period of 1 min was adopted; for stability constant determinations, a period of 5 min was adopted. At the end of the equilibrium period, the spectrum of the solution was then recorded. The cycle was repeated automatically until the defined end point pH value was achieved. All of the titration data were analyzed with the pHab program.[22] The species plot was calculated with the HYSS program.[23] Analytical grade reagent materials were used in the preparation of all solutions.

Determination of Cell Survival. *Cell Culture.* Three different human carcinoma cell lines, Hela, A549, and MCF-7, were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 mg/mL of streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Samples were dissolved in DMSO at a concentration of 10 mM as stock solution, and diluted with PBS buffer to concentrations of 100, 10, and 1 μM, then diluted with DMEM medium to final concentrations of 10, 1, and 0.1 μM as working solutions. To avoid DMSO toxicity, the concentration of DMSO was less than 0.1% (v/v) in all experiments.

Cytotoxicity Analysis. The cells harvested from exponential phase (2×10^4 cells/mL, 100 μL) were seeded into a 96-well plate. Cells were allowed to completely adhere overnight, and then the supernatant was removed. Fresh DMEM medium (90 μL) was complemented, and then the samples (10 μL) were added to the wells. The incubation was continued for 48 h. Control wells were prepared by addition of culture medium. Wells containing culture medium without cells were used as blanks. All experiments were performed in quintuplicate. The MTT assay was performed as described by Mosmann.[24] Upon completion of the incubation, stock MTT dye solution (10 μL, 5 mg/mL) was added to each well. After 4 h incubation, the supernatant was removed, and DMSO (100 μL) was added to dissolve the MTT formazan. The OD of each well was measured on a microplate spectrophotometer at a wavelength of 570 nm.

Statistical Analyses. All experiments were performed in triplicate. The data were statistically analyzed using analysis of variance test (SPSS version 13.0). Data in the tables and figure were given as mean ± SD and analyzed by one-way ANOVA. Significant differences between the treatments were examined by Duncan's multiple range test. $P < 0.05$ was considered as statistically significant.

■ RESULTS AND DISCUSSION

Synthesis of Hydroxypyridinone-L-phenylalanine Conjugates (5). Compounds **5** were synthesized starting from kojic acid (**1**) (Scheme 1). Benzylation of 5-hydroxy group in kojic acid was achieved by the reaction of kojic acid and benzyl chloride under basic condition to provide **2** in good yield. Condensation of **2** with amine generated **3**, which was then coupled with Cbz-L-phenylalanine by ester bond formation in the presence of EDC and DMAP in *N,N*-dimethylformamide (DMF) at room temperature, yielding product **4**. Deprotection of the benzyl and Cbz groups on **4** was achieved by hydrogenation in the presence of palladium/charcoal, generating hydroxypyridinone-L-phenylalanine conjugates **5**. All of the compounds have been fully characterized by ^{1}H NMR, MS, and HRMS.

Inhibitory Effect of Compounds 5 on Monophenolase Activity of Tyrosinase. Tyrosinase inhibitory activities of the hydroxypyridinone-phenylalanine conjugates were evaluated by means of the mushroom tyrosinase inhibition assay method. Using L-tyrosine as substrate, the inhibitory effects on tyrosinase increased with the increase of concentrations of **5** (Figure 1). Statistical analysis of the results indicated that a

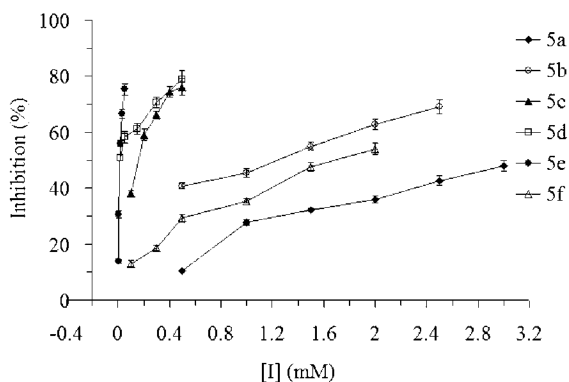
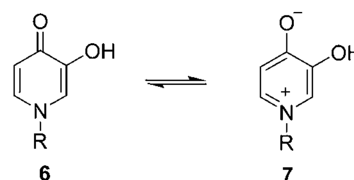


Figure 1. Inhibitory effects of hydroxypyridinone-phenylalanine conjugates on monophenolase activity of mushroom tyrosinase. The assays were carried out in triplicates under the following reaction conditions: pH 6.8, 30 °C, 10 min.

significant difference was observed between the uses of different inhibitors ($P < 0.05$). For each inhibitor, the significant difference in inhibitory effect also occurred between the each tested concentration ($P < 0.05$). IC_{50} values were summarized in Table 1. Compounds **5e** was found to exhibit potent inhibitory activity against tyrosinase with an IC_{50} value of 12.6 μM, which was lower than that of kojic acid (IC_{50} = 26.8 μM). From compounds **5a** to **5e**, the inhibitory effect increased with the increase of hydrophobicity of the compound, which is in good agreement with previously published data.[6] However, **5f**, the most hydrophobic compound with the highest $C \log P$ value[25] of 4.707 (Table 1), was found to possess a weak inhibitory activity. This is probably due to the fact that **5f** has a poor water solubility. Thus, for the hydroxypyridinone derivatives to exert a good tyrosinase inhibitory effect, it is essential for the compounds to possess a suitable hydrophobicity.

Although some reported kojic acid derivatives possess higher tyrosinase inhibitory activity, for example, Kim et al. reported the synthesis of a kojic acid derivative with a potent tyrosinase inhibitory activity (IC_{50} = 3.63 μM),[26] it needs longer synthetic procedure. As compared to pyranone ring in kojic acid derivatives, pyridinone ring in hydroxypyridinone derivatives is more stable because it possesses aromaticity due to the existence of tautomerism **7** (Scheme 2). In comparison with the reported

Scheme 2. Tautomerization of Hydroxypyridinone Derivatives



hydroxypyridinone derivatives (the lowest IC_{50} value is 0.37 mM),[27] **5e** exhibited stronger tyrosinase inhibitory activity.

Inhibitory Effect of 5d and 5e on Diphenolase Activity of Tyrosinase. The inhibitory activity of **5e** and **5d** on the diphenolase activity of mushroom tyrosinase was investigated using L-DOPA as a substrate. The IC_{50} values of **5d** and **5e** on the diphenolase activity of the enzyme were determined as 42.8 and 4.0 μM, respectively (Table 1). Thus, **5e** also exhibited a potent inhibitory effect on the diphenolase activity of mushroom tyrosinase greater than kojic acid (IC_{50} = 20 μM).[28] The kinetic data of the inhibition of L-DOPA oxidation by **5e** and **5d** were expressed in a nonlinear Michaelis–Menten plot (Figure 2A-I, B-I) and Lineweaver–Burk double-reciprocal plots (Figure 2A-II, B-II). As can be seen in the nonlinear Michaelis–Menten plot, V_{max} decreased with the increase of inhibitor concentration, whereas K_m increased with the increase of inhibitor concentration, indicating that **5d** and **5e** can bind not only with free enzyme but also with the enzyme–substrate complex. This result can also be obtained from Lineweaver–Burk double-reciprocal plots. As is shown in Figure 2A-II and B-II, the intersection of the lines in the second quadrant indicated that both **5e** and **5d** were competitive–uncompetitive mixed-type inhibitors, suggesting that both compounds could bind with both free enzyme and the enzyme–substrate complexes (ES). The equilibrium constant for inhibitor binding with free enzyme (K_I) was obtained from a plot of slope (K_m/V_m) versus the concentration of the inhibitor, and with ES (K_{IS}) was obtained from a plot of the vertical intercept ($1/V_m$)

Table 1. Inhibitory Effect of Hydroxypyridinone-L-phenylalanine Conjugates (5) against Monophenolase and Diphenolase Activity of Mushroom Tyrosinase

compounds	$C \log P$	IC_{50} (μM) (against monophenolase activity)	IC_{50} (μM) (against diphenolase activity)	K_I (μM)	K_{IS} (μM)
5a	0.238	2590 ± 55.8			
5b	0.614	1150 ± 31.5			
5c	1.676	190 ± 6.4			
5d	2.686	19.2 ± 0.8	42.8 ± 2.3	55.5 ± 1.5	193.5 ± 12.5
5e	3.697	12.6 ± 0.3	4.0 ± 0.1	4.0 ± 0.03	8.36 ± 0.53
5f	4.707	1650 ± 41.3			
kojic acid	−0.888	26.8 ± 1.2	20	13.0	100.0

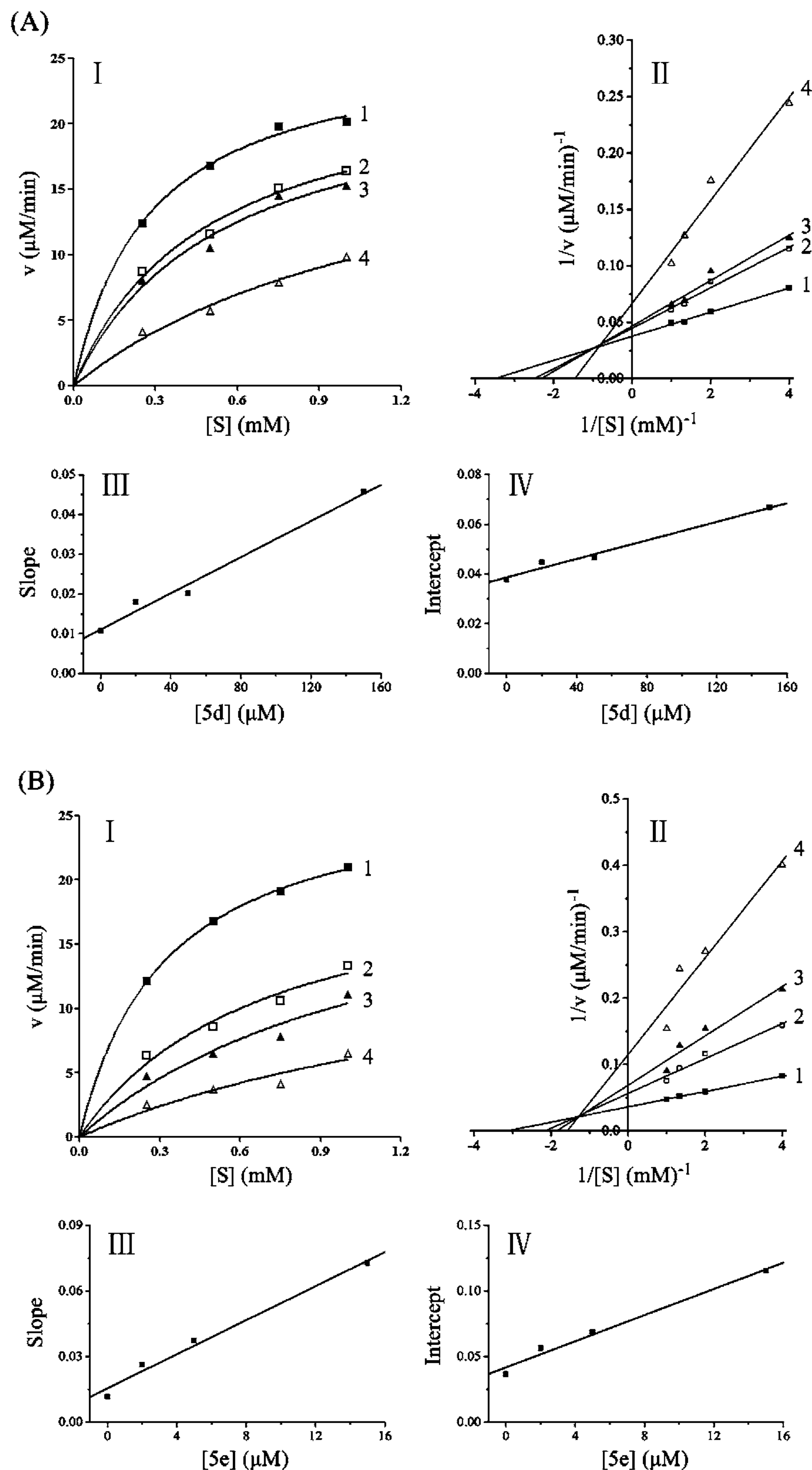
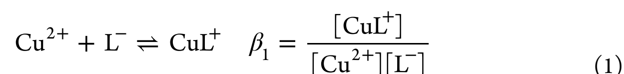


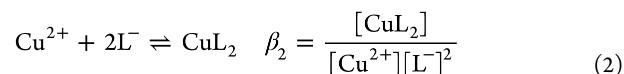
Figure 2. Nonlinear Michaelis–Menten plot (A-I and B-I) and Lineweaver–Burk plots (A-II and B-II) of mushroom tyrosinase with L-DOPA as a substrate in the presence of **5d** (A) and **5e** (B). A-I and A-II: The concentrations of **5d** for curves 1–4 were 0, 20, 50, and 150 μM, respectively. B-I and B-II: The concentrations of **5e** for curves 1–4 were 0, 2, 5, and 15 μM, respectively. A-III and B-III represent the plot of slope versus the concentration of **5d** and **5e** for determining the inhibition constants K_I. A-IV and B-IV represent the plot of intercept versus the concentration of **5d** and **5e** for determining the inhibition constants K_{IS}.

versus the concentration of the inhibitor. The K_I and K_{IS} values of **5d** were determined to be 55.5 and 193.5 μM, respectively. The inhibitor constants (K_I and K_{IS}) of **5e** were measured as 4.0 and 8.36 μM, respectively, which are lower than those of kojic acid (K_I = 13.0 μM, K_{IS} = 100.0 μM).[28] In both cases, the K_{IS} value is larger than the K_I value, indicating that the affinity of the inhibitors for free enzyme is greater than that for the enzyme−substrate complex.

pK_a Values and Copper Affinity of 5e. To explore the inhibitory mechanism of **5e** on tyrosinase, the pK_a values and the affinity for copper were evaluated. The pH dependence UV spectra of **5e** (Figure 3) was recorded between 250 and 350 nm over the pH range 1.88−11.11 for the free ligand. The speciation spectra demonstrate a clear shift in λ_{max} from 280 to 310 nm, which reflects the pH dependence of the ligand ionization equilibrium. Using the spectrophotometric titration method, the pK_a values of **5e** obtained from nonlinear least-squares regression analysis were found to be 3.191 ± 0.06, 8.776 ± 0.07, and 10.34 ± 0.05, which correspond to the 4-oxo function, the 5-hydroxyl function, and the amino group, respectively. The stability constant of a metal−ligand complex is one of the key parameters related to the chelation efficacy of a ligand. As a bidentate, **5e** can form two species of copper complexes, CuL^+ and CuL_2. The stability constants of these two metal−ligand complexes can be expressed as follows:



$$Cu^{2+} + L^- \rightleftharpoons CuL^+ \quad \beta_1 = \frac{[CuL^+]}{[Cu^{2+}][L^-]} \tag{1}$$



$$Cu^{2+} + 2L^- \rightleftharpoons CuL_2 \quad \beta_2 = \frac{[CuL_2]}{[Cu^{2+}][L^-]^2} \tag{2}$$

where L^- represents ligand.

Employing spectrophotometric titration against the hydroxyl anion, the log stability constants of the two complexes (CuL^+ and CuL_2), log β_1 and log β_2, were determined to be 9.294 ± 0.09 and 15.719 ± 0.11, respectively. A series of UV spectra of **5e** in the presence of copper at different pH values is shown in Figure 4. As the pH increased from 2, the absorbance at 700 nm gradually rose, and then the peak shifted to 650 nm. This indicates the formation of the copper complexes CuL^+ and CuL_2 correspondingly.

Other synthesized compounds (**5a**−**5d** and **5f**) were assumed to possess similar affinities for copper, which is higher than that of kojic acid (log β_1 = 6.6, log β_2 = 11.7).[29] However, with the exception of **5e** and **5d**, other compounds exhibited a lower activity than kojic acid. It is possible that the hydrophobic alkyl chain at the N-1 position plays an important role in the inhibitory activity of **5e** and **5d**. Hydrophobic interaction of the hydrophobic alkyl chain with the hydrophobic protein pocket surrounding the binuclear copper active site in the enzyme may disrupt tyrosinase's quaternary structure.[30]

The cytotoxicity of **5e** and kojic acid on Hela, A549, and MCF-7 cell lines was examined using a MTT assay. From the assay results, it was found that **5e** was slightly less toxic than kojic acid overall, lacking obvious toxicity up to 10 μM (Table 2).

In conclusion, a range of hydroxypyridinone-L-phenylalanine conjugates were synthesized. Among them, **5e** exhibited higher tyrosinase inhibitory activity than did kojic acid. Moreover, **5e** was demonstrated to be nontoxic to three cell lines, and also could find application in the cosmetic and food preserving areas.

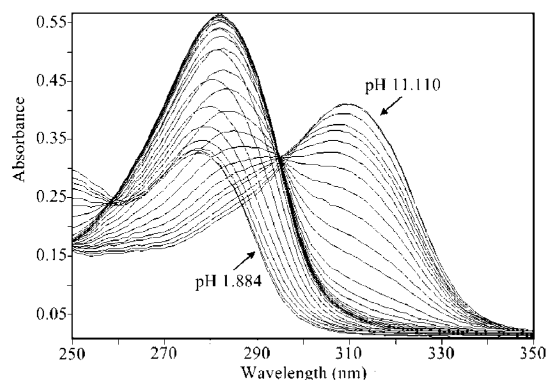


Figure 3. UV spectra of **5e**. [**5e**] = 870 μM (in 20.120 mL of 0.1 M KCl), pH was changed from 1.884 to 11.110 by the addition of KOH at 25 °C.

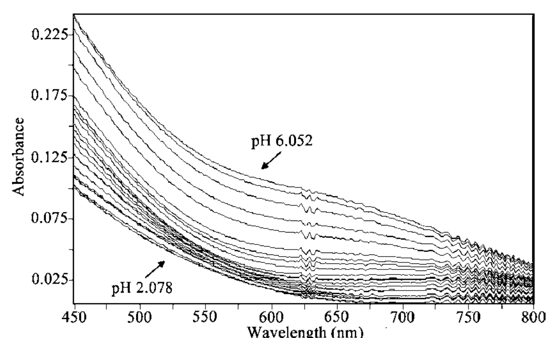


Figure 4. UV spectra of **5e** in the presence of copper. [**5e**] = 814.6 μM, [Cu^{2+}] = 237 μM (in 20.414 mL of 0.1 M KCl), pH was changed from 2.078 to pH 6.052 by the addition of KOH at 25 °C.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

DMAP, 4-dimethylaminopyridine; EDC, *N*-(3-dimethylamino-propyl)-*N*′-ethylcarbodiimide hydrochloride; DMF, *N*,*N*-dimethylformamide; ES, enzyme−substrate complexes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Table 2. Cell Survival Results Based on MTT Assay (%)

	5e (μM)			kojic acid (μM)		
cell lines	0.1	1.0	10	0.1	1.0	10
Hela	104.2 ± 2.7	104.8 ± 3.6	101.4 ± 3.7	102.7 ± 2.2	104.6 ± 3.9	102.2 ± 3.4
A549	97.7 ± 3.1	97.0 ± 2.2	100.1 ± 2.8	99.3 ± 2.5	99.7 ± 2.6	98.9 ± 3.2
MCF-7	96.7 ± 1.9	102.0 ± 2.4	99.6 ± 2.2	97.8 ± 3.1	96.5 ± 2.7	92.3 ± 2.6

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