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Tyrosinase inhibitory effects and antioxidative activities of novel cinnamoyl amides with amino acid ester moiety

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ABSTRACT

Nine cinnamoyl amides with amino acid ester (CAAE) moiety were synthesized by the conjugation of the corresponding cinnamic acids (cinnamic acid, 4-hydroxy cinnamic acid, ferulic acid and caffeic acid) with amino acid esters, and their inhibitory effects on the activities of mushroom tyrosinase were investigated, using L-3,4-dihydroxyl-phenylalanine (L-DOPA) as the substrate. Among these CAAE amides, ethyl *N*-[3-(4-hydroxy-3-methoxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate (**b**$_4$) showed the strongest inhibitory activity; the IC_{50} was 0.18 μM. The IC_{50} values, inhibition types, inhibition mechanisms and kinetics of all these CAAE amides were evaluated. A structure–activity relationship (SAR) study found that the inhibitory effects were potentiated with the increasing length of hydrocarbon chains at the amino acid esters and also influenced by the substituents at the styrene groups. Furthermore, the hydroxyl radical scavenging and anti-lipid peroxidation activities of four CAAE derivatives were also investigated. Among these compounds, **b**$_3$ (ethyl *N*-[3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate) and **b**$_4$ exhibited potential antioxidant activities.

1. Introduction

Pigmentation is one of the most obvious phenotypical characteristics in the natural world. Enzymatic browning in plants, animals and microorganisms is a typical pigmentation, which is catalyzed by a copper-containing enzyme, tyrosinase (EC.1.14.18.1; also known as catecholase or diphenol oxidase) (Huang et al., 2006; Mayer, 1995). Tyrosinase is a multifunctional enzyme that catalyzes both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones (Lee, 2002). Tyrosinase also plays an important role in the biosynthesis of melanin (Okombi et al., 2006), which widely spreads in skin, hair and eyes of mammals. On the other hand, melanin may involve in pigmentation-related disorders of human. Moreover, the melanin-related browning can cause undesirable quality loss in colour, flavour and nutrition of food products, which may reduce their commercial values. The degree of browning among fruits and vegetables is variable because of the differences in the phenolic content and tyrosinase activity (Martinez & Whitaker, 1995; Shi, Chen, Wang, Song, & Qiu, 2005). Therefore, the inhibitors of tyrosinase are of great importance in preventing browning of food products and inhibiting the synthesis of melanin in medicinal and cosmetic products. Development of tyrosinase inhibitors as food additives and cosmetic products has interested more and more researchers, and many novel tyrosinase inhibitors have been discovered (Huang et al., 2006; Yi, Wu, Cao, Song, & Ma, 2009).

The inhibitory effects of hydroxylated cinnamic acid derivatives (caffeic acid, ferulic acid, and *p*-coumaric acid) on tyrosinase have been reported extensively (Gómez-Cordovés, Bartolomé, Vieira, & Virador, 2001; Lee, 2002). Interestingly, some *N*-hydroxycinnamoylphenalkylamides exhibited better inhibitory activities on mushroom tyrosinase than cinnamic acid did (Okombi et al., 2006; Shi et al., 2005). Furthermore, some amide derivatives of these acids are naturally present in plants (Roh, Han, Kim, & Hwang, 2004). Yoon-Sik Lee reported the tyrosinase inhibitory activities of Kojic acid and hydroxyphenolic acid derivatives modified with amino acids, and found that compounds with phenylalanine moiety showed the strongest inhibitory activities (Noh & Lee, 2011; Noh et al., 2009).

Inspired by these findings, two series of cinnamyl amides of amino acid ester (CAAE) (series **a** and **b**, see Fig. 1 for structures) were synthesized and their inhibitory effects on mushroom tyrosinase were studied. Furthermore, the antioxidative activities of series **b** compounds against hydroxyl radical and lipid peroxidation were also investigated.

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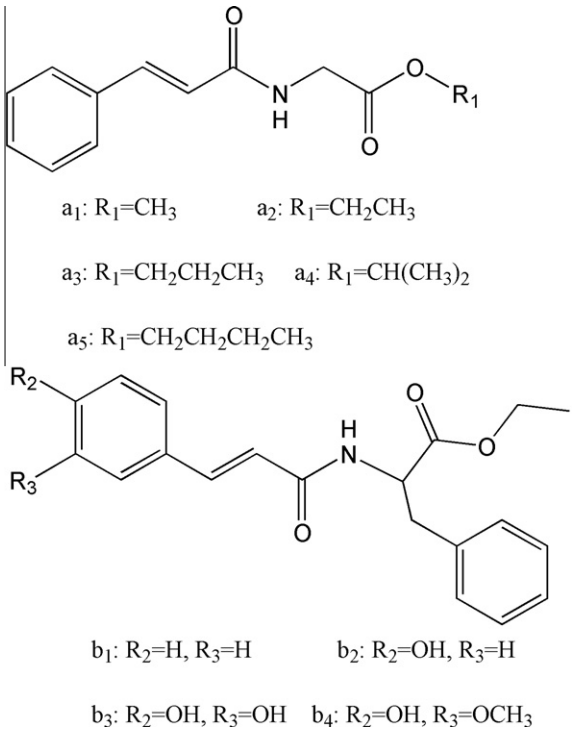

Fig. 1. Chemical structures of CAAE derivatives.

2. Materials and methods

2.1. Chemical reagents and instruments

IR spectra were recorded on an Avatar 330 infra-red spectrophotometer (KBr pellet); only the most significant absorption bands were reported (ν_{max}, cm^{-1}). Melting points were determined on a RY-2 melting apparatus. ^1H NMR data were acquired at room temperature on a Bruker AV 400-MHz operating at 400 MHz or 300-MHz operating at 300 MHz. $CDCl_3$ or DMSO-d_6 was used as solvent. Chemical shifts were expressed in δ (parts per million) values relative to tetramethylsilane (TMS) as internal reference. Mass was performed on a Saturn 2000 mass spectrometer. A Spectronic Genesys 8 UV/Vis spectrophotometer was used in the antioxidative assays.

Caffeic acid, ferulic acid, *p*-coumaric acid, mushroom tyrosinase (EC.1.14.18.1), 3,4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from Sigma (St. Louis, MO, USA). 1-Hydroxybenzotriazole (HOBt), 1-[3-(dimethylamino)propyl]-3-ethylcarbo diimidehydrochloride (DEC·HCl), and dimethylsulphoxide (DMSO) were purchased from Shanghai Medpep Co., Ltd., China. All other reagents were of analytical grade. The water used was re-distilled and ion-free.

2.2. Synthesis

Nine CAAE derivatives were synthesized from cinnamic acid or substituted cinnamic acids and the corresponding amino acid esters, according to literature with some modifications (Orlandi, Rindone, Molteni, Rummakko, & Brunow, 2001). The synthesis method was exemplified by the preparation of compound $\mathbf{b}_3$. Generally, a solution of 3,4-dihydroxy cinnamic acid (1.0 g, 5.5 mmol), L-ethyl phenylalaninate hydrochloride (0.75 g, 5.5 mmol), and 1-hydroxybenzotriazole (0.74 g, 5.5 mmol) in *N*,*N*-dimethylformamide (15 ml) was added to triethylamine (2.3 ml, 16.5 mmol) at 0 °C. After 10 min, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimidehydrochloride (EDC·HCl 1.0 g, 5.5 mmol) was added at 0 °C and the resultant reaction mixture was stirred for 18 h at room temperature. The mixture was then poured into water (100 ml), extracted with ethyl acetate (4 × 100 ml), washed with water (2 × 100 ml) and saturated brine (100 ml), and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residual paste was purified by column chromatography (silica gel, hexane/EtOAc (1:1)) to give ethyl *N*-[3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate ($\mathbf{b}_3$). Other compounds were synthesized in the similar way with compound $\mathbf{b}_3$. The structures of the synthesized compounds were established by IR, ^1H NMR and EIMS. The results were as follows:

Methyl N-[3-phenyl-1-oxo-2-propen-1-yl] glycinate ($\boldsymbol{a}_1$): Yield 90.2%, white solid, melting point: 121–122 °C; ^1H NMR (300 MHz, $CDC1_3$): 7.64 (d, 1H, J = 15.6 Hz, HC=C), 7.62–7.25 (m, 5H, C_6H_5), 6.46 (d, 1H, J = 15.6 Hz, C=CH), 6.22 (br s, 1H, NH), 4.19 (d, 2H, J = 4.8 Hz, CH_2), 3.79 (s, 3H, OCH_3); IR (KBr) υ, cm^{-1}: 3297.3 (N—H), 3033.4 (Ar—H), 3008.0 (C=C—H), 1726.2 (C=O), 1650.6 (O=C—N), 1612.5 (C=C), 1222.5 (C—O—C); MS (*m*/*z*, %): 219.9 ($M^{\cdot+}$, 100.0), 130.8 (45.3).

Ethyl N-[3-phenyl-1-oxo-2-propen-1-yl] glycinate ($\boldsymbol{a}_2$): Yield 89.5%, white solid, melting point: 102–104 °C; ^1H NMR (400 MHz, $CDC1_3$): 7.65 (d, 1H, J = 15.6 Hz, HC=C), 7.53–7.37 (m, 5H, C_6H_5), 6.47 (d, 1H, J = 15.6 Hz, C=CH), 6.15 (br s, 1H, NH), 4.25 (q, 2H, J = 7.6 Hz, OCH_2), 4.19 (d, 2H, J = 4.8 Hz, CH_2), 1.31 (t, 3H, J = 7.6 Hz, CH_3); IR (KBr) υ, cm^{-1}: 3362.4 (N—H), 3083.4 (Ar—H), 3035.9 (C=C—H), 1747.8 (C=O), 1655.3 (O=C—N), 1625.7 (C=C), 1074.9 (C—O—C); MS (*m*/*z*, %): 233.9 ($M^{\cdot+}$, 100.0), 130.8 (12.3).

Propyl N-[3-phenyl-1-oxo-2-propen-1-yl] glycinate ($\boldsymbol{a}_3$): Yield 94.3%, white solid, melting point: 66–67 °C; ^1H NMR (400 MHz, $CDC1_3$): 7.67 (d, 1H, J = 15.6 Hz, HC=C), 7.62–7.36 (m, 5H, C_6H_5), 6.44 (d, 1H, J = 15.6 Hz, C=CH), 6.19 (br s, 1H, NH), 4.19 (t, 2H, J = 7.8 Hz, CH_2), 4.15 (d, 2H, J = 4.0 Hz, CH_2), 1.65–1.70 (m, 2H, CH_2), 0.96 (t, 3H, J = 7.8 Hz, CH_3); IR (KBr) υ, cm^{-1}: 3287.7 (N—H), 3061.6 (Ar—H), 3027.6 (C=C—H), 1750.0 (C=O), 1661.4 (O=C—N), 1623.1 (C=C), 1208.2 (C—O—C); MS (*m*/*z*, %): 247.9 ($M^{\cdot+}$, 100.0), 130.8 (9.7).

Isopropyl N-[3-phenyl-1-oxo-2-propen-1-yl] glycinate ($\boldsymbol{a}_4$): Yield 89.7%, white solid, melting point: 108–110 °C; ^1H NMR (400 MHz, $CDC1_3$): 7.66 (d, 1H, J = 15.6 Hz, HC=C), 7.52–7.37(m, 5H, C_6H_5), 6.47 (d, 1H, J = 15.6 Hz, C=CH), 6.14 (br s, 1H, NH), 5.08–5.14 (m, 1H, CH), 4.15 (d, 2H, J = 4.8 Hz, CH_2), 1.29 (d, 6H, J = 6.4 Hz, $2CH_3$); IR (KBr) υ, cm^{-1}: 3448.1 (N—H), 3059.8 (Ar—H), 3002.3 (C=C—H), 1758.6 (C=O), 1701.4 (O=C—N), 1634.93 (C=C), 1075.1 (C—O—C); MS (*m*/*z*, %): 247.9 ($M^{\cdot+}$, 100.0), 130.8 (9.5).

Butyl N-[3-phenyl-1-oxo-2-propen-1-yl] glycinate ($\boldsymbol{a}_5$): Yield 83.6%, white solid, melting point: 93–94 °C; ^1H NMR (400 MHz, $CDC1_3$): 7.64 (d, 1H, J = 15.6 Hz, CH=C), 7.52–7.37(m, 5H, C_6H_5), 6.43 (d, 1H, J = 15.6 Hz, C=CH), 6.04 (br s, 1H, NH), 4.78 (t, 2H, J = 4.0 Hz, CH_2), 4.16 (d, 2H, J = 4.8 Hz, —CH_2), 1.67–1.59 (m, 2H, CH_2), 1.42–1.37(m, 2H, CH_2—CH3), 0.98 (t, 3H, J = 6.4 Hz, —CH3); IR (KBr) υ, cm^{-1}: 3382.5 (N—H), 3060.2 (Ar—H), 3027.0 (C=C—H), 1768.0 (C=O), 1701.6 (O=C—N), 1632.5 (C=C), 1207.7 (C—O—C); MS (*m*/*z*, %): 261.9($M^{\cdot+}$, 100).

Ethyl N-[3-phenyl-1-oxo-2-propen-1-yl]-L-phenylalaninate ($\boldsymbol{b}_1$): Yield 90.3%, white solid, melting point: 79–80 °C; ^1H NMR (400 MHz, $CDC1_3$): 7.64 (d, 1H, J = 15.6 Hz, HC=C), 7.52–7.12 (m, 10H, C_6H_5), 6.43 (d, 1H, J = 15.6 Hz, C=CH), 6.14 (br s, 1H, NH), 5.01(q, 1H, J = 4.0 Hz, CH), 4.23–4.18 (m, 2H, CH_2), 3.21 (q, 2H, J = 7.2 Hz, CH_2), 1.27 (t, 3H, J = 7.2 Hz, CH_3); IR (KBr) υ, cm^{-1}: 3224 (N—H), 3061 (Ar—H), 3034 (C=C—H), 1739 (C=O), 1654 (O=C—N), 1626 (C=C), 1209 (C—O—C); MS (*m*/*z*, %): 325.0 ($M^{\cdot+}$, 100.0).

Detailed characterization data of the compounds ethyl *N*-[3-(4-hydroxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate ($\mathbf{b}_2$),

ethyl *N*-[3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate (**b**$_3$), and ethyl *N*-[3-(4-hydroxy-3-methoxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate (**b**$_4$) were listed in our previous paper (Wei, Jiang, Zhang, Guo, & Wang, in press).

2.3. Enzyme activity assay

The spectrophotometric assay for diphenolase activity of mushroom tyrosinase was performed according to the method reported by Yi et al. (2009) with slight modifications. In brief, L-DOPA was used as substrate. All compounds were dissolved in dimethylsulphoxide (DMSO) and the final concentration of DMSO in the test solution was less than 2.0% (v/v). First, 10 μl (0.5 mg/ml) mushroom tyrosinase with the indicated concentrations of compounds was pre-incubated in 980 μl 0.1 M K_2HPO_4–KH_2PO_4 buffer (pH 6.8) for 10 min at 25 °C. Ten microlitres of 0.26 mM L-DOPA were then added to initiate the reaction. The enzyme activities were determined by following the increase of absorbance at 475 nm for 1 min with a molar absorption coefficient of 3700 (M^{-1} cm^{-1}), accompanying the oxidation of L-DOPA to dopachrome. The measurement was performed in triplicate for each concentration and averaged before further calculation. The inhibition type was assayed by Lineweaver–Burk plot.

2.4. Determinations of antioxidative activity of **b**$_1$, **b**$_2$, **b**$_3$ and **b**$_4$

2.4.1. Preparation of rat brain homogenate

The preparation was carried out according to the method reported by Wei, Chen, Zhou, Yang, and Liu (2006) with some modifications. In brief, female Wistar rats weighing 250 ± 20 g were starved overnight and sacrificed by cervical dislocation under ether anaesthesia. Then the brain was rapidly removed and washed extensively with 0.15 M NaCl. The tissue sample was homogenized with 10 ml of ice-cold 0.15 M KCl, 10 mM Tris–HCl buffer, pH 7.4. Later, the tissue homogenate was centrifuged at 800*g* for 10 min to remove the residues. The supernatant was collected and stored at −20 °C. The protein content of brain homogenate was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.4.2. Anti-lipid peroxidation assay

Anti-lipid peroxidation activity of the four compounds **b**$_1$, **b**$_2$, **b**$_3$ and **b**$_4$ were evaluated. The formation of thiobarbituric acid-reactive substance (TBARS) was used to monitor the lipid peroxidation product malondialdehyde (MDA), which was used as an indicator of lipid peroxidation (Wei et al., 2006). Rat brain homogenate was made up to a final protein concentration of 0.4 mg/ml in buffer (10 mM Tris–HCl, 0.15 M KCl, pH 7.4). Antioxidants were dissolved in DMSO and added to 0.5 ml of rat brain homogenate, in which the final concentration of DMSO solution was less than 0.2% (v/v) that did not show appreciable interference to the reaction. Then, 5 μl of 10 mM ascorbic acid and 2 μl of 2.5 mM $FeCl_2$ were added to the foresaid brain homogenate to initiate lipid peroxidation. The reaction mixture was incubated for 1 h at 37 °C in a capped tube. After incubation, 0.75 ml TBA reagent (15% TCA–0.375% TBA–0.25 M BHT) was added and shaken vigorously. The mixture was heated in a boiling water bath for 15 min. After cooling, the precipitate was removed by centrifugation. TBARS in the supernatant was determined at 532 nm using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.4.3. Hydroxyl radical-scavenging activity assay

The assay of hydroxyl radical-scavenging activity of the four samples was carried out according to a previous research (Yi et al., 2009), with minor changes. Briefly, 100 μl of ethanolic solution of 7.5 mM phenanthroline, 100 μl of aqueous solution of 7.5 mM $FeSO_4$, 50 μl of sample solution (dissolved in DMSO) and 650 μl of 50 mM phosphate buffer (pH = 7.4) were mixed in a test tube. Then, 100 μl of H_2O_2 solution (dissolved in deionised water) was added to the tube. As the control, 50 μl of DMSO was added instead of sample solution to the tube. The mixture was left at 37 °C in the dark for 1 h. Finally, the absorbance was measured at 536 nm.

2.5. Statistical analysis

Data were presented as mean ± standard deviations (SD) from three independent analyses. All analyses were carried out with the origin statistical and analysis software.

3. Results

3.1. Inhibition of tyrosinase activity by CAAE derivatives

The inhibitory effects on mushroom tyrosinase activities for the oxidation of L-DOPA by nine CAAE derivatives (see Fig. 1 for structures), cinnamic acid and Kojic acid (as positive control) were probed. All of the nine compounds have inhibitory effects on the activities of the tyrosinase. The enzyme activities decreased dramatically with the increasing concentrations of the tested compounds (data not showed). In order to compare the inhibitory potencies among them, the IC_{50} values, which stand for the concentrations leading to a 50% activity loss of tyrosinase, of the nine tested compounds that were determined. The results were summarized and shown in Table 1. Among the nine compounds, the IC_{50} values of compounds a$_3$, **a**$_4$, **a**$_5$, and **b**$_{1-4}$ were less than 100 μM. Compounds **a**$_5$ and **b**$_4$ were the most potent inhibitors, while compounds **a**$_1$ and **a**$_2$ were the weakest ones. For the series **a**, the inhibitory effects followed the sequence: **a**$_1$ < **a**$_2$ < **a**$_3$ < **a**$_4$ < **a**$_5$, and the inhibitory effects were potentiated with the increasing lengths of the hydrocarbon chains at the amino acid esters. And for the series **b**, the inhibition strength followed the order: **b**$_1$ ≈ **b**$_2$ < **b**$_3$ < **b**$_4$, with different substituted groups at the styrene groups.

3.2. The inhibitory effects of compounds **a**$_3$, **a**$_4$ and **a**$_5$ on mushroom tyrosinase following a non-competitive mechanism

The inhibitory types of compounds **a**$_3$, **a**$_4$ and **a**$_5$ on mushroom tyrosinase, were determined during the oxidation of L-DOPA from Lineweaver–Burk plots (Kubo & Kinst-Hori, 1999; Noh & Lee, 2011). Fig. 2 shows the double-reciprocal plots of the enzyme inhibited by compound **a**$_3$. The plots of $1/v$ versus $1/[S]$ gave a family of straight lines with different slopes, but they intersected one another at the horizontal axis. The enhancement of the inhibitor concentration could decrease the values of V_{max}, but the values of K_m remained the same, which indicated that **a**$_3$ was a non-competitive inhibitor for the mushroom tyrosinase. This behaviour showed that **a**$_3$ could bind with both free enzyme and enzyme–substrate (ES) complex, and their equilibrium constants were the same. The inhibition constants for the inhibitor binding with the free enzyme (K_I) and enzyme–substrate (ES) complex (K_{IS}) were determined by the plot of vertical intercept ($1/V_{max}$) versus the inhibitor concentrations, which was a line as showed in the inset of Fig. 2. Compounds **a**$_4$ and **a**$_5$ were studied with the same method and similar results were obtained (data not showed). The inhibition constants were summarized in Table 1 and the results showed that both of them were also non-competitives.

3.3. The inhibitory effects of compounds **b**$_1$, **b**$_2$, **b**$_3$ and **b**$_4$ on mushroom tyrosinase following a mixed-type non-competitive mechanism

The inhibitory mechanisms of compounds **b**$_1$, **b**$_2$, **b**$_3$ and **b**$_4$ on mushroom tyrosinase were also studied from Lineweaver–Burk

Table 1
Inhibitory effects and constants of mushroom tyrosinase by CAAE derivatives.

Compounds	IC_{50} (μM)	Inhibition type	Inhibition constants (μM)	
			K_I	K_{IS}
a_1	460 ± 20	ND	ND	ND
a_2	257.5 ± 12.5	ND	ND	ND
a_3	20 ± 2	Non-competitive	40	
a_4	14.5 ± 1.5	Non-competitive	1.25	
a_5	1.8 ± 0.2	Non-competitive	1.43	
b_1	74 ± 8.5	Mixed-type	30	193
b_2	80 ± 20	Mixed-type	24	110
b_3	31.2 ± 1.9	Mixed-type	9.15	5.64
b_4	0.185 ± 0.005	Mixed-type	0.011	0.076
Kojic acid	22 ± 2.45	ND	ND	ND
Cinnamic acid	475 ± 65			

The IC_{50} values represent means ± SD of three parallel experiments. ND: not determination.

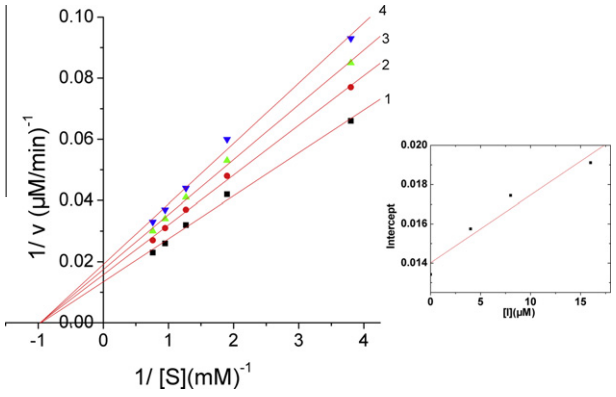

Fig. 2. Lineweaver–Burk plots for inhibition of $\mathbf{a}_3$ on mushroom tyrosinase for the catalysis of L-DOPA. Concentrations of $\mathbf{a}_3$ for curves 1–4 were 0, 4, 8 and 16 μM, respectively. The inset represents the secondary plot of $1/V_{max}$ versus concentrations of $\mathbf{a}_3$ to determine the inhibition constant.

plots (Noh & Lee, 2011). Fig. 3 shows the double-reciprocal plots of the enzyme inhibited by compound $\mathbf{b}_1$. The curves with different slopes and intercepts intersect one another in the second quadrant. This behaviour indicated that $\mathbf{b}_1$ could bind with both free enzyme and enzyme–substrate (ES) complex and their equilibrium constants were different, which indicated that $\mathbf{b}_1$ acted as a competitive–uncompetitive mixed-I type inhibitor (Noh & Lee, 2011). The inhibition equilibrium constants for the inhibitor binding with

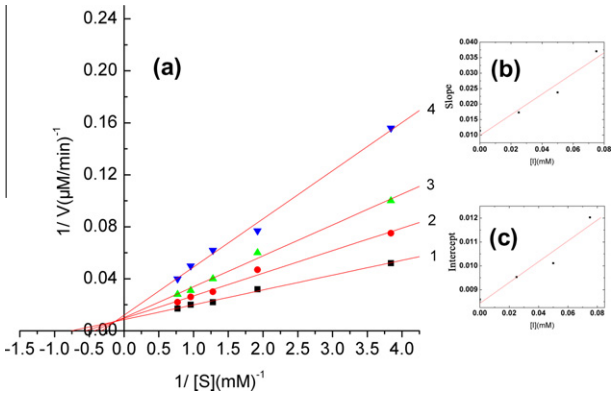

Fig. 3. Lineweaver–Burk plots for inhibition of $\mathbf{b}_1$ on mushroom tyrosinase for the catalysis of L-DOPA. Concentrations of $\mathbf{b}_1$ for curves 1–4 were 0, 0.025, 0.05 and 0.075 mM, respectively. Insets (b) and (c) represent the secondary plots of the slopes and the intercepts versus concentrations of $\mathbf{b}_1$ to determine the inhibition constants.

the free enzyme, K_I, and with ES, K_{IS}, were determined by the secondary plots of K_m/V_m and $1/V_{max}$ versus the inhibitor concentrations (inset in Fig. 3), respectively. Both of the secondary plots are linear. Similar results were obtained with $\mathbf{b}_2$ and b_4 and their inhibition constants were summarized in Table 1. From the determined values, we noticed that the K_I values were lower than K_{IS}, indicating that the affinity of the inhibitor for the free enzyme was stronger than that for the enzyme–substrate complex. The value of K_{IS} was about five times greater than that of K_I, indicating that the competitive effect was stronger than the uncompetitive effect.

The inhibitory type of $\mathbf{b}_3$ on mushroom tyrosinase was determined by the same methods. The results showed that with the increasing concentration of $\mathbf{b}_3$, a family of lines with different slopes and intercepts intersect one another in the third quadrant (Fig. 4). K_I and K_{IS} were obtained from the secondary plots by the same methods mentioned above and summarized in Table 1. In this case, the K_{IS} values were lower than K_I, which indicated that $\mathbf{b}_3$ was a mixed-II type inhibitor (Chen et al., 2005).

3.4. Antioxidative activity of compounds $\mathbf{b}_1$, $\mathbf{b}_2$, $\mathbf{b}_3$ and $\mathbf{b}_4$

The inhibitory effects of CAAE derivatives ($\mathbf{b}_1$, $\mathbf{b}_2$, $\mathbf{b}_3$ and $\mathbf{b}_4$) on TBARS production in rat brain homogenates induced by $FeCl_2$–ascorbic acid were shown in Fig. 5. Caffeic acid was taken as a positive control. The results indicated that when the compounds were of the same concentration (80 μM), $\mathbf{b}_3$ showed the strongest antioxidative activity among the four compounds. Its inhibition ratio of TBARS production was 88.7%, approaching the level of caffeic acid, 93.4%. And $\mathbf{b}_4$ came in the second place, with the inhibition ratio of 50%. The compounds $\mathbf{b}_1$ and $\mathbf{b}_2$ had the weakest antioxidative activities, with the inhibition ratios of TBARS production 16% and 34.6%, respectively. The IC_{50} values of the compounds $\mathbf{b}_1$, $\mathbf{b}_2$, $\mathbf{b}_3$ and $\mathbf{b}_4$ for the hydroxyl radical-scavenging activity, were also determined. The IC_{50} values of the compounds $\mathbf{b}_1$, $\mathbf{b}_2$ were more than 2 mM, while the IC_{50} values of the compounds $\mathbf{b}_3$, and $\mathbf{b}_4$ were 0.13 and 0.58 mM, respectively. The scavenging strength followed the order: $\mathbf{b}_1 < \mathbf{b}_2 < \mathbf{b}_4 < \mathbf{b}_3 <$ caffeic acid. The combination of these antioxidative activity results showed that, $\mathbf{b}_3$ and $\mathbf{b}_4$ were good antioxidants against hydroxyl radical, and that rat brain homogenates lipid peroxidation was induced by hydroxyl radical.

4. Discussion

Wine phenolic compounds, such as caffeic acid, ferulic acid, and *p*-coumaric acid have been reported to act as tyrosinase inhibitors (Gómez-Cordovés et al., 2001). Aromatic carboxylic acids of cinnamic acid and their analogues, *p*-coumaric, ferulic, and sinapic

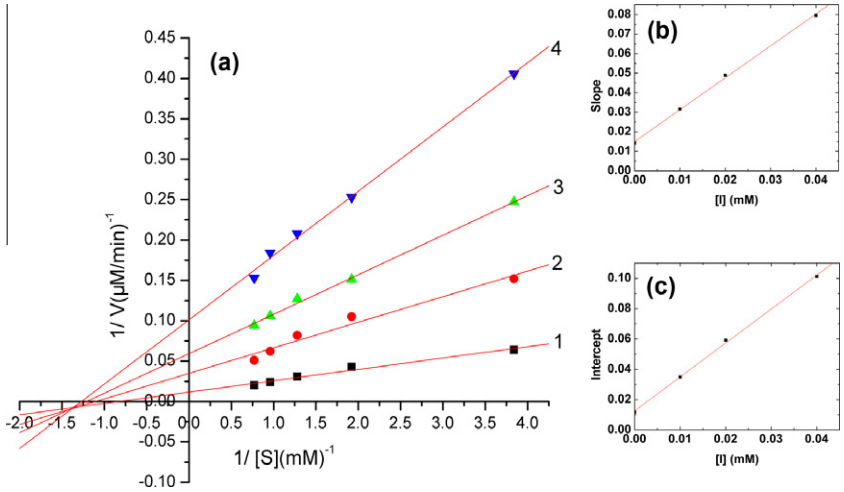

Fig. 4. Lineweaver–Burk plots for inhibition of $\mathbf{b}_3$ on mushroom tyrosinase for the catalysis of L-DOPA. Concentrations of $\mathbf{b}_3$ for curves 1–4 were 0, 0.01, 0.02 and 0.04 mM, respectively. Insets (b) and (c) represent the secondary plots of the slopes and the intercepts versus concentrations of $\mathbf{b}_3$ to determine the inhibition constants.

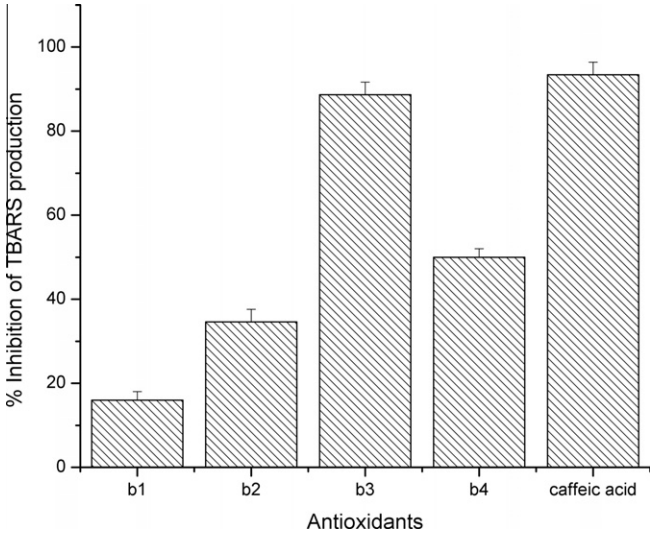

Fig. 5. Inhibitory effects of different CAAE derivatives ($\mathbf{b}_1$, $\mathbf{b}_2$, $\mathbf{b}_3$ and $\mathbf{b}_4$) on lipid peroxidation in rat brain homogenates induced by $FeCl_2$–ascorbic. The concentrations of the inhibitors are 80 μM, respectively. Data were presented as the percentage of inhibition on the lipid peroxidation, means ± SD (n = 3).

acids are competitive inhibitors of polyphenoloxidase (PPO) due to their structural similarities to the phenolic substrates (Kim, Marshall, & Wei, 2000). Nirmal and Benjakul (2009) reported the effects of ferulic acid (FA) on polyphenoloxidase (PPO) of Pacific white shrimp (*Litopenaeus vannamei*) and the concentration of 50% inhibition was about 1% (w/v) (Nirmal & Benjakul, 2009). Furthermore, cinnamic acid and its derivatives were inhibitors of tyrosinase (Qiu et al., 2009; Shi et al., 2005) and their IC_{50} values were estimated to be more than 0.4 mM for the oxidation of L-DOPA initiated by mushroom tyrosinase. In this study, several cinnamic acid derivatives (cinnamic acid, 4-hydroxy cinnamic acid, ferulic acid and caffeic acid) were modified by conjugating with amino acid esters, and their effects on the oxidation of L-DOPA initiated by mushroom tyrosinase were screened. Among the nine modified cinnamyl amino acid esters, the IC_{50} values of $\mathbf{a}_5$ and $\mathbf{b}_4$ was less than 2 μM and 0.2 μM, respectively, which were more effective inhibitors of diphenolase activity than Kojic acid (Table 1). As shown in Table 1, each of the nine cinnamoyl amino acid ester (CAAE) derivatives revealed an enhanced inhibitory activity compared with cinnamic acid.

Huang et al. (2006) reported that *p*-octylbenzoic acid was the most activity one in the inhibition activity of mushroom tyrosinase by alkylbenzoic acid. Moreover, they concluded that the inhibitory effects were potentiated with the increasing lengths of the hydrocarbon chains. The present work assesses the inhibitory activities of mushroom tyrosinase of nine cinnamoyl amino acid ester (CAAE) derivatives, and enables a structure–activity relationship (SAR) of these compounds. As Table 1 showed, among the **a** series the $\mathbf{a}_1$–$\mathbf{a}_5$ compounds bear different lengths of hydrocarbon chains at the amino acid esters. Compounds $\mathbf{a}_1$ and $\mathbf{a}_2$ which bear methoxy- and ethoxy- at the amino acid ester respectively were less active ones (IC_{50} > 200 μM), while $\mathbf{a}_3$, $\mathbf{a}_4$ and $\mathbf{a}_5$ which bear propoxy-, isopropoxy- and butoxy- at the amino acid ester, respectively, were more active ones. Noteworthily, $\mathbf{a}_5$ was the most active. It is suggested that the inhibitory activities of the CAAE derivatives on the diphenolase activity of mushroom tyrosinase were enhanced with the increasing lengths of hydrocarbon chains at the amino acid esters. Similarly, when cinnamic acid was modified with phenylalanine ($\mathbf{b}_1$) instead of glycine ($\mathbf{a}_2$), the inhibitory effects were also enhanced. When Kojic acid and hydroxyphenolic acid derivatives were modified with amino acids, the compounds with phenylalanine moiety were found to have the strongest inhibitory activities of mushroom tyrosinase (Noh & Lee, 2011; Noh et al., 2009). Butoxy- side chain at the amino acid ester and the phenylalanine ($\mathbf{b}_1$) residue may contribute to the hydrophobic effect of these compounds, which could increase the hydrophobic interactions with the hydrophobic side chains located at the tyrosinase active site (Noh & Lee, 2011). The substituents at the styrene groups were also important for the activity when the activities of $\mathbf{b}_3$ and $\mathbf{b}_4$were compared with that of $\mathbf{b}_2$ or $\mathbf{b}_1$ as Noh and Lee (2011) found for the inhibitory activities of hydroxyphenolic acid–amino acid conjugates on tyrosinase. However, in our study, when a methoxyl group appeared in the meta-position (compared $\mathbf{b}_4$ with $\mathbf{b}_3$), the inhibitory effect was strengthened significantly. The difference in the inhibitory activities between these two compounds ($\mathbf{b}_4$ and $\mathbf{b}_3$) could be explained by the lower electron-donating capacity of methoxy- compared with hydroxy- at the styrene group.

Recently, the crystallographic structure of tyrosinase has been reported, enabling a closer look at its three-dimensional structure and a better understanding of its mechanism of action (Khatib et al., 2007; Matoba, Kumagai, Yamamoto, Yoshitsu, & Sugiyama, 2006; Noh & Lee, 2011; Noh et al., 2009). Within the structures, there are two copper ions in the active centre of tyrosinase and a

lipophilic long-narrow gorge near to the active centre. Our results showed that all of the CAAE derivatives were reversible inhibitors and they could be mainly divided into two groups: **a** series, including $\mathbf{a}_3$, $\mathbf{a}_4$ and $\mathbf{a}_5$, were non-competitive inhibitors, while **b** series which included $\mathbf{b}_1$, $\mathbf{b}_2$, $\mathbf{b}_3$ and $\mathbf{b}_4$ were mixed-type inhibitors. Shi et al. (2005) summarized the inhibitory types of cinnamic acid and its derivatives on tyrosinase and they found that cinnamic acid was non-competitive, while 4-hydroxy cinnamic acid was competitive. In our research, when cinnamic acid was modified with glycine ester ($\mathbf{a}_3$, $\mathbf{a}_4$ and $\mathbf{a}_5$), their inhibitory types were the same as that of cinnamic acid. We suggest that the modification of cinnamic acid by conjugating with glycine ester ($\mathbf{a}_3$, $\mathbf{a}_4$ and $\mathbf{a}_5$) may help insert the tested compounds into the lipophilic long-narrow gorge near to the active centre, which may hinder the binding of the substrate to the enzyme through steric hindrance or by changing the protein conformation. Interestingly, when cinnamic acid, 4-hydroxy cinnamic acid, ferulic acid and caffeic acid were conjugated with phenylalanine ester (Table 1), they became mixed-type inhibitors. As for $\mathbf{b}_1$, $\mathbf{b}_2$ and $\mathbf{b}_4$, the value of K_{IS} was about five times greater than that of K_I, indicating that the binding of inhibitors to the free enzyme was stronger than to the enzyme–substrate complex, which may be induced by hydrophobic interaction near the active site of tyrosinase. Noh et al. (2009) docked Kojic acid–amino acid conjugates into the active site of tyrosinase using the AUTODOCK Tools program, and found that quite a few hydrophobic amino acids were located around the copper active site of tyrosinase. The fact that hydrophobic interactions existed between the aromatic rings of Kojic acid–amino acid conjugates and the hydrophobic side chains in the tyrosinase active site was confirmed, and it was concluded that these interactions blocked the accessibility of the substrate to the active site (Noh & Lee, 2011; Noh et al., 2009). Our results were similar to these reports. The different inhibition types of **b** series compounds from **a** series may be attributed to the aromatic rings of phenylalanine. However, in the **b** series, compound $\mathbf{b}_3$ was a different one. Compound $\mathbf{b}_3$ could bind with the enzyme–substrate complex more easily and tightly, which may be induced by the hydrogen-bonding interactions between the two adjacent positions of hydroxyls and the amino groups in the tyrosinase. A previous study reported that hydrogen-bonding interactions could also influence the stability of the oxy-form of tyrosinase (Kubo & Kinst-Hori, 1999). The inhibition mechanism of compound $\mathbf{b}_3$ needs to be more studied.

Free radical mediated peroxidation of membrane lipids and oxidative damage of DNA and proteins are believed to be associated with a variety of chronic health problems, such as cancer, atherosclerosis, neurodegenerative diseases and ageing (Wei et al., 2006). Hydroxy cinnamic acid is one class of phenolic compounds and hydroxycinnamates are phenylpropanoid metabolites, which occur widely in plants and plant products (Clifford, 1999). Hydroxycinnamates and their derivates exhibit *in vitro* antioxidant activity, which might be beneficial to health (Kroon & Williamson, 1999). It has been reported that caffeic acid phenylethyl ester (CAPE) has an ameliorating effect on the oxidative stress via its antioxidant property in cardiac tissue (Okutana, Ozcelikb, Ramazan Yilmaz, & Uz, 2005). Furthermore, in food industry, oxidation is one of the most important processes involved in food deterioration because it may affect food qualities (colour, flavour and texture), leading to the quality loss of products. Antioxidants may help preserve food quality by preventing the oxidative deterioration of lipids (Kinsella, Frankel, German, & Kanner, 1993). In our continuing research for antioxidants (Wei et al., in press), $\mathbf{b}_3$ and $\mathbf{b}_4$ showed good antioxidative activities among the four different substituted cinnamamides (cinnamoyl, caffeoyl, feruloyl, and *p*-coumaroyl). Analyzing the inhibitory effects and their molecular structures, we concluded that with the increasing number of hydroxyl groups attached to the benzene ring, the anti-lipid peroxidation of homogenates activity induced by hydroxyl radical increased accordingly. The conclusion was identical with what Yi et al. (2009) summarized in their study.

In summary, in this paper, nine cinnamoyl amino acid ester derivatives, **a** series and **b** series were designed, synthesized and their inhibitory effects on mushroom tyrosinase were investigated. Their inhibition mechanisms and inhibition kinetics were also studied. Furthermore, their antioxidant activities of hydroxyl radical scavenging and anti-lipid peroxidation of homogenates induced by hydroxyl radical were also revealed. All the data suggested that $\mathbf{b}_3$, $\mathbf{b}_4$, and $\mathbf{a}_5$ might serve as new potent preservatives for the food industry or skin-whitening agents in cosmetics. Further development of such compounds may be of interest.

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