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Structure–activity relationships of antityrosinase and antioxidant activities of cinnamic acid and its derivatives

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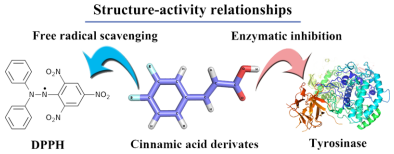
ABSTRACT

The related structure–activity relationships of cinnamic acid and its derivates have not been studied in details yet. Herein, antityrosinase and antioxidant activities of 18 compounds were evaluated. The results demonstrated that the substituents on the phenyl ring of cinnamic acid led to the enhancement of the inhibition on monophenolase and the weakening of the inhibition on diphenolase. Among these tested compounds, **9** was first discovered as a tyrosinase inhibitor in a reversible competitive manner with IC_{50} value of 68.6 ± 4.2 μM. Docking results demonstrated **9** located into the catalytic center of tyrosinase. Antioxidant assay indicated that only 1 hydroxyl group on the phenyl ring was not enough to possess the radical scavenging activity, and the number of hydroxyl groups may be more important. This study will be helpful in the development of new cinnamic acid derivates as tyrosinase inhibitors and antioxidants with higher efficacy.

Graphical Abstract



The structure–activity relationships of antityrosinase and antioxidant activities of cinnamic acid and its derivatives have been analyzed in this study.

Keywords: structure–activity relationships, antityrosinase, antioxidant

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Tyrosinase (E.C. 1.14.18.1) is a multifunctional metalloenzyme and widely distributed in microorganisms, plants and animals (Sánchez-Ferrer *et al.* 1995). It has the catalytic centers formed by binuclear copper that catalyze 2 distinct reactions involving the hydroxylation of monophenols (monophenolase activity) and oxidation of o-diphenols to the corresponding o-quinones (diphenolase activity) (Khan *et al.* 2005; Asthana *et al.* 2015). These o-quinones could polymerize spontaneously to form macromolecular dark or brown pigments through a series of reactions such as oxidations and polymerizations (Kashima and Miyazawa 2013). Thus, tyrosinase as the rate limiting enzyme in the reactions mentioned above plays a crucial role in multiple physiological and pathological pathways. In humans, the abnormal activity of tyrosinase may overproduce melanin that can cause some dermatological disorders including freckles, senile lentigines and melasma; besides, tyrosinase has also been found to be linked to neurodegenerative diseases such as Huntington's, Alzheimer's, and Parkinson's diseases recently (Song *et al.* 2017). In foods, tyrosinase is responsible for enzymatic browning that impacts the quality of fresh-cut fruits and vegetables, such as nutritive and commercial value loss (Friedman 1996). In insects, tyrosinase is involved in the molting process, wound healing and parasite encapsulation and thus considered as a new target for the control of insect pests (Shiino, Watanabe and Umezawa 2001). Therefore, extensive attentions have been paid to search for safe and effective tyrosinase inhibitors that may have potential applications in the medicinal, cosmetic, food, and agricultural industries.

Cinnamic acid, an unsaturated carboxylic acid, distributes in a number of plants such as cinnamon and styrax (Sheng *et al.* 2018). It and its derivatives are a class of phenolic acids and widely distributed in vegetables, fruits and whole grains, and nowadays they can be obtained from either natural or synthetic source (Adisakwattana 2017). It has been found that cinnamic acid and its derivatives have various pharmacological activities, including antibacterial, antifungal, anti-inflammatory, antioxidant and anticancer activities (Garcia Jimenez *et al.* 2018). Recently, they have also been found to have the ability to inhibit the activity of tyrosinase *in vitro* (Takahashi and Miyazawa 2010). Besides, the antimelanogenic effects of cinnamic acid and its derivatives on B16F10 melanoma cells have been reported (Nazir *et al.* 2020). Moreover, the kinetic analysis of the activity of tyrosinase inhibited by cinnamic acid and some of its derivatives have been done (Shi *et al.* 2005; Cui *et al.* 2017). Though these studies may have provided clues for rational design of potent tyrosinase inhibitors, it may be more valuable to clarify the structure–activity relationships (SARs) of the inhibition of tyrosinase by cinnamic acid and its derivatives. Preliminary SARs analysis displayed that conjugated double bond of cinnamic acid and its derivatives is an essential group for tyrosinase inhibition (Takahashi and Miyazawa 2010), and hydroxylation and methoxylation on the phenyl ring may be important factors determining their inhibitory activities (Zhang *et al.* 2013). However, a great deal of experimental researches is still needed to be carried out to further clarify the SARs in details. In addition, it has been found that several cinnamic acid derivatives have strong antioxidant activities that are strongly related to their structural features (Wendy, Cuvelier and Berset 1995), but the SARs for the antioxidant activities of them has not been well discussed.

Thus, the SARs of cinnamic acid and its derivatives from natural or synthetic source (totally 18 compounds) inhibiting tyrosinase and scavenging free radical were analyzed in this study. Notably, some of the compounds tested here have not been discovered as tyrosinase inhibitors before. Herein, the effects of cinnamic acid and its derivatives on both monophenolase and diphenolase activities were investigated by using L-tyrosine and L-dopa as the substrate, respectively. The SARs were discussed in details based on the IC_{50} (the half-maximal inhibitory concentration) values of these compounds that obtained from the relative enzymatic activity versus the concentration of inhibitor curves. Among the tested 18 compounds, trans-3,4-difluorocinnamic acid (**9**) was first identified as the most potential inhibitor, and thus the kinetics, inhibition mechanism and molecular docking were carried out to further clarify its inhibitory effects on tyrosinase. Additionally, the free radical scavenging abilities of cinnamic acid derivatives were evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the SARs for the antioxidant activities was also discussed. All the results in this manuscript may help to design and develop new efficient tyrosinase inhibitors and antioxidants and push the application of cinnamic acid derivatives in medicinal, cosmetic, food and agricultural industries.

Materials and methods

Materials

Cinnamic acid and its derivatives (analytical grade) were all purchased from Aladdin Bio-technology (Shanghai, China), and the number (Compound No.) and the name of these compounds were shown in Table 1. Mushroom tyrosinase (EC 1.14.18.1), L-tyrosine and L-dopa were purchased from Sigma-Aldrich (St. Louis, MO, USA). DPPH, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), kojic acid and ascorbic acid were also obtained from Aladdin Bio-technology (Shanghai, China). All other chemicals used were analytical or pharmaceutical grade. A Milli-Q-Plus ultrapure water system from Millipore (Sartorius

Table 1. Cinnamic acid and its derivatives

Compound no.	Name	Compound no.	Name
1	Cinnamic acid	10	Caffeic acid
2	4-Fluorocinnamic acid	11	3,4-Dimethoxycinnamic acid
3	4-Chlorocinnamic acid	12	Ferulic acid
4	4-Bromocinnamic acid	13	Isoferulic acid
5	4-Nitrocinnamic acid	14	Trans-3-hydroxycinnamic acid
6	4-Coumaric acid	15	Methyl cinnamate
7	4-Methylcinnamic acid	16	α-Methylcinnamic acid
8	4-Methoxycinnamic acid	17	3-Phenylpropionic acid
9	Trans-3,4-difluorocinnamic acid	18	3-(4-Hydroxyphenyl)propionic acid

611, Germany) was used throughout the study to obtain water used during the experiments.

Evaluation of antityrosinase activity

The tyrosinase inhibitory assays were performed according to our previously reported method with a few modifications (Chen, Yu and Huang 2016). The stock solution of each compound (20 mM) was prepared by using DMSO as solvent and doubly diluted to different concentrations for assay. 100 μL of compound solution (negative or positive control sample) was added to 2.8 mL of L-tyrosine (0.5 mM, for monophenolase assay) or L-dopa (0.5 mM, for diphenolase assay) and well-mixed. After incubation for 5 min at 305 K, 100 μL of tyrosinase (1000 U/mL for monophenolase assay and 400 U/mL for diphenolase assay) was added to the mixture to start the reactions. The absorbance of each sample was then measured every 1 min for 20 min at 475 nm by detecting the formation of dopachrome with UV–vis spectrophotometer (UV 2550, SHIMADZU). Then the absorbance versus time curve was plotted, from which the initial reaction rate (v, slope of the line) of each reaction was calculated. Thus, the relative enzymatic activity was calculated by the following equation: Relative enzymatic activity (%) = v_i (reaction rate in the presence of inhibitor)/v_0 (reaction rate in the absence of inhibitor) × 100%. The half maximal inhibitory concentration (IC_{50}) was obtained from the plot of enzymatic activity versus the concentration of the tested compound. Each sample was analyzed at least 3 times and the results were averaged. The solvent DMSO and kojic acid were used as negative and positive control, respectively.

Kinetic analysis for competitive-type inhibition

Among the tested compounds, **9** (trans-3,4-difluorocinnamic acid) exhibited the highest inhibitory activity, and hence, we carried out the kinetic analysis to determine the type of inhibition. The protocol of the experiment was the same as mentioned above except for using different concentrations (0.25-2.0 mM) of L-dopa as the substrate. The compound solutions at final concentrations (0-666.67 μM) were also prepared. Tyrosinase with a constant concentration (400 U/mL) was used in all the experiments. The initial velocities of the reactions (v), in the absence and presence of inhibitors, were obtained from the linear portion of the time curves of catalytic reaction of tyrosinase. And then the linear regression plot of $1/v$ versus 1/[S] (the concentrations of substrate) can be drawn, and the values of slope were obtained. The inhibition constant (K_i) was obtained from the second plots of slope versus [I] (the concentration of the inhibitor), and the type of inhibition was confirmed by Lineweaver–Burk. More specifically, for competitive type inhibition, the Lineweaver–Burk equation can be expressed in double reciprocal form as reported before (Chen *et al.* 2020b).

Molecular docking

In order to further explore the interaction between **9** and tyrosinase, molecular docking studies were performed according to our previous study with some modifications by using AutoDock (4.2.6) (Chen, Yu and Huang 2016). The three-dimensional (3D) structure of tyrosinase (PDB ID: 2Y9X) was retrieved from the RCSB Protein Data Bank, while the 3D structure of **9** and cinnamic acid (reference compound) were constructed by ChemBio3D Ultra 14.0. A grid box (60 Å × 60 Å × 60 Å) was defined to enclose the catalytic center of tyrosinase with a grid spacing of 0.375 Å. Docking calculations were performed using the Lamarckian Genetic Algorithm and the search parameters were set to 100 times. The conformation of the compound with the lowest docking energy was chosen to represent its most favorable binding mode, and then it was visually analyzed by using the PyMOL and Ligplot$^+$ (1.4.5) software.

DPPH radical scavenging assay

The DPPH radical scavenging activity of compounds was determined using the method that we reported previously with some modifications (Chen *et al.* 2018). In brief, the DPPH solution (0.5 mM) was appropriately diluted with ethanol to make its absorbance of 0.7 ± 0.02 units at the wavelength of 517 nm. 2.0 mL of diluted DPPH was pipetted into a cuvette, and then 100 μL of compound solution (0.625-20 mM) was added and well mixed. The mixture was kept at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm using UV–vis spectrophotometer (UV 2550, SHIMADZU). The experiments were repeated at least 3 times at each separate concentration of the tested compounds. Ascorbic acid was used as a reference compound. Ethanol was used as a negative control, the value of which was subtracted from the values of the tested compounds. The percentage inhibition (%) of absorbance at 517 nm can be calculated by the following equation: inhibition (%) = $[1 - (A_1 - A_3)/A_2] \times 100\%$, where A_1 is the absorbance in the presence of tested compound and DPPH radical, A_2 is the absorbance in the presence of equal ethanol and DPPH radical, and A_3 is the absorbance in the presence of tested compound and equal ethanol. Then the EC_{50} (concentration required to obtain a 50% antioxidant effect or inhibition of absorbance) was obtained from the plot of the percentage inhibition versus the concentration of the tested compounds.

Statistical analysis

All the experiments were repeated at least 3 times and the results were averaged. Data were collected and analyzed by using Excel (version 2007, Microsoft) and SPSS (version 12, IBM) software. Data were compared using one-way ANOVA and expressed as mean ± SD. The p-value of 5% or lower was considered to be statistically significant.

Results and discussion

SARs of inhibition on monophenolase activity

The structures of the 18 tested compounds were shown in Figure 1, and 1 stands for cinnamic acid. Compounds **2-8** are 4-substituted (on the phenyl ring) cinnamic acid derivatives with substituents including fluorine (2), chlorine (3), bromine (4), nitro (5), hydroxyl (6), methyl (7), and methoxy (8). Some of them were tested for the effects on tyrosinase for the first time, such as **2** and **4**. The results were summarized in Table 2. Compared **2-4** with cinnamic acid, it was found that halogen substituent enhanced the inhibitory effects on monophenolase. Specifically, the inhibitory effects (**2** > **3** > **4**) increased with the electronegative values of halogen (F > Cl > Br). Compound **2** with fluorine substituent has the strongest inhibitory activity with IC_{50} value of 132 ± 6.1 μM, which was 5 times more active than cinnamic acid (658 ± 22 μM). The results suggested that the presence of halogen substituent on the 4-position significantly enhanced the inhibitory activity, the intensive of which may be involved in the electronegative values of the substituent.

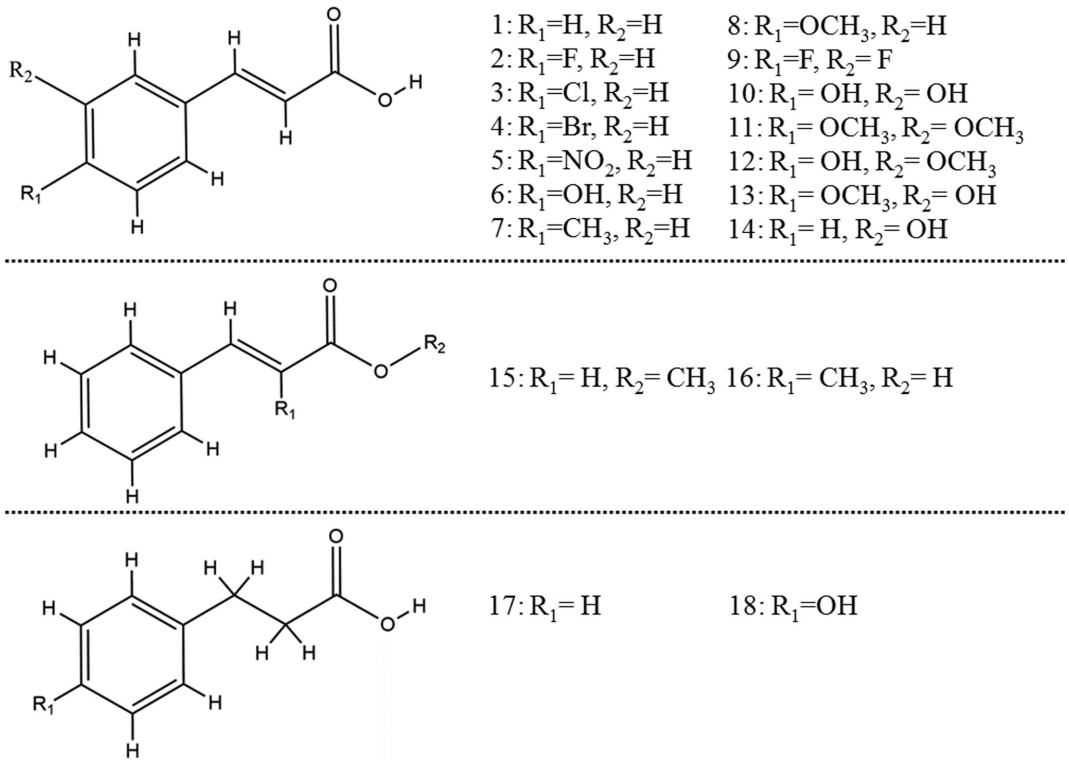

Figure 1. Structures of cinnamic acid and its derivatives (**1-18**).

Table 2. Inhibitory effects of **1-18** on tyrosinase

Compound	Monophenolase IC_{50} (μM)[a]	Diphenolase IC_{50} (mM)[a]	Compound	Monophenolase IC_{50} (μM)[a]	Diphenolase IC_{50} (mM)[a]
1	658 ± 22	0.98 ± 0.06	**10**	NA[b]	NA[b]
2	132 ± 6.1	1.65 ± 0.05	**11**	NA[b]	3.07 ± 0.11
3	195 ± 14	2.13 ± 0.03	**12**	243 ± 6.2	NE[b]
4	564 ± 16	1.71 ± 0.03	**13**	84.8 ± 5.3	5.76 ± 0.16
5	316 ± 5.7	1.92 ± 0.05	**14**	NA[b]	NA[b]
6	232 ± 4.2	NA[b]	**15**	857 ± 10	4.84 ± 0.24
7	878 ± 23	2.43 ± 0.12	**16**	1.23×10^3 ± 21	6.13 ± 0.13
8	624 ± 15	1.97 ± 0.08	**17**	2.71×10^3 ± 27	NA[b]
9	68.6 ± 4.2	0.78 ± 0.02	**18**	NA[b]	NA[b]
Kojic acid	7.76 ± 0.56	0.10 ± 0.01			

[a]Values were obtained from the relative enzymatic activity versus the concentration of inhibitor curves and expressed as means ± SD ($n = 3$).
[b]Not available (NA) at the experimental conditions.

The inhibitory activities of **1**, **5-8** decreased in the following order: –OH > $–NO_2$ > $–OCH_3$ > –H > $–CH_3$, demonstrating once again that hydroxyl plays an important role in enhancing the inhibitory activity (Huang *et al.* 2006). These compounds except for **7** had more potent activity than cinnamic acid. This confirmed once again that hydroxyl or methoxy on the 4-position of the phenyl ring help to enhance the inhibitory activity (Billaud, Lecornu and Nicolas 1996). The above results indicated that the addition of substituent on the 4-position of the phenyl ring may enhance the inhibitory activity, which was consisted with the previous work (Cui *et al.* 2017). Notably, the substituents on the 4-position described here are not exactly the same as the previously reported, suggesting that it may be a universal law.

Compounds **9-13** are cinnamic acid derivatives bearing 2 substituents on 3- and 4-positions respectively of the phenyl ring. Among these compounds, **9** with 2 fluorine substituents exhibited the strongest inhibitory activity with IC_{50} value of 68.57 ± 4.23 μM. Compared to **2** (132 ± 6.1 μM), the results indicated that the fluorine group on the 3-position of the phenyl ring further enhanced the inhibitory activity. The presence of hydroxyl (**10**) or methoxy (**11**) substituent on both 3- and 4-positions of the phenyl ring led to undetectable tyrosinase inhibitory activity, compared to **6** and **8**, suggesting an additional hydroxyl or methoxy on 3-position decreased the inhibitory activity. This result further confirmed that the increase of hydroxyl number on the phenyl ring did not necessarily lead to the enhancement of inhibitory activity (Wu *et al.* 2012). When compared **8** with **11** and **13**, the hydroxyl group on 3-position enhanced the inhibitory activity, while the methoxy group on the same position decreased the inhibitory activity. The results are consisted with the previous report (Takahashi and Miyazawa 2010). Compound **12** is also called ferulic acid bearing hydroxyl

and methoxy substituents on 4- and 3-positions respectively of the phenyl ring, while **13** is its isomer, named isoferulic acid bearing methoxy and hydroxyl substituents on 4- and 3-positions respectively of the phenyl ring. Isoferulic acid with IC_{50} value of 84.8 ± 5.3 μM showed much higher inhibitory activity than ferulic acid with IC_{50} value of 243 ± 6.2 μM. The small difference in the structure of **12** and **13** led to tremendous difference in their inhibitory activities, the reason of which may be worth to be further explored and it will be helpful for the development and utilization of cinnamic acid derivatives. Compound **14** with hydroxyl substituent on 3-position showed no inhibitory activity, compared to **6** and **1**, indicating the position of hydroxyl on the phenyl ring plays a big role in affecting the inhibitory activity and excessive hydroxyl may even lead to the loss of inhibitory activity due to steric hindrance (Kim *et al.* 2006). From the differences among the inhibitory activities of **10-14**, it was found that the effects of substituents (on 3- and 4-positions) on the inhibitory activity were complicated, which may be the result of coaction of electrostatic repulsion and steric hindrance effect.

In the case of **15**, the esterification of carboxyl group could compromise the inhibitory activity relative to cinnamic acid. This is consisted with the previous report that the esterification of the carboxylic group decreased the inhibitory activity (Pifferi, Baldassari and Cultrera 1974). Similarly, the presence of methyl in the double bond (**16**) negatively influenced the inhibitory activity. The double bond of cinnamic acid derivatives may be an essential group for inhibitory activity when compared **17** with **1**, which was further confirmed by comparing **18** with **6**. This result was consisted with the previous report, which may be attributed to the spatial interaction between the double bond moiety and the tertiary structure of tyrosinase catalytic center when the carboxylic acid part is coordinated with the catalytic center of the enzyme (Takahashi and Miyazawa 2010). Although some of the SARs provided here have been reported before, the tested compounds were not exactly the same, so they could be confirmed and supplemented by each other. The SARs of cinnamic acid derivates inhibiting monophenolase activity may be helpful to design new tyrosinase inhibitor with higher efficacy.

SARs of inhibition on diphenolase activity

We also evaluated the inhibitory activity of these compounds by taking L-dopa as a substrate. As shown in Table 2, the IC_{50} values of each compound on diphenolase were significantly higher than that on monophenolase, suggesting that each tested compound exhibited more potent inhibitory effects on monophenolase than diphenolase. The result was consisted with the previous report (Lim, Ishiguro and Kubo 1999), and this is possibly due to the fact that there are 2 independent binding sites in the catalytic center of tyrosinase, one is for the binding of monophenols (monophenolase activity) and the other is for the binding of diphenols (diphenolase activity) (Pomerantz and Warner 1967). And thus the structure of cinnamic acid and its derivatives may be more potent to match or disturb the conformation of the catalytic center for binding monophenols. Therefore, the SARs of cinnamic acid derivatives inhibiting tyrosinase may also be different when taking monophenols or diphenols as substrates.

From the results of diphenolase inhibitory activity, it was found that almost all compounds had much weaker inhibitory activity than cinnamic acid, and the only exception was **9** with 2 fluorine substituents on 3- and 4-positions, respectively. This result suggested that the substituents of cinnamic acid generally led to the decrease of inhibitory effects on diphenolase activity. Specifically, compared **2-8** with cinnamic acid, it was found that the substituents on 4-position decreased the inhibitory effects on diphenolase activity. It was worth noting that **6** as a substrate did not exhibited inhibitory effects on diphenolase activity despite the strong inhibitory effects on monophenolase activity, which was consisted with the previous report described that the hydroxyl group on the 4-position increased monophenolase inhibitory activity while decreasing diphenolase inhibitory activity (Lim *et al.* 1999). And this further confirmed that there were different catalytic centers for monophenols and diphenols substrates (Pomerantz and Warner 1967). Moreover, unlike the monophenolase activity, there were no obvious regular relationships between the substituents of halogen on the phenyl ring and the inhibitory activity.

Compared **10** and **11** with cinnamic acid, it was found that the substitute of methoxy group contributed little to decreasing the diphenolase activity than that of hydroxyl group. The position of methoxy and hydroxyl substituents on the phenyl ring may play a key role in the inhibitory activity, according to the fact that both the monophenolase and diphenolase inhibitory activity of **12** were much lower than that of **13**. The similar result has been reported by Zhang et al. as well (Zhang *et al.* 2013). No obvious diphenolase inhibitory effect of **14** demonstrated that the hydroxyl group on the 3-position also deceased the inhibitory activity. The diphenolase inhibitory activity of **15** and **16** were lower than cinnamic acid, suggesting that either the esterification of the carboxylic group or the presence of methyl in the double bond negatively influenced the inhibitory activity. Compared **17** with cinnamic acid, it was also found that the presence of double bond was essential for the diphenolase inhibitory activity. In summary, it was first discovered that the SARs of cinnamic acid derivates inhibiting monophenolase and diphenolase are totally different, confirming that cinnamic acid derivates generally preferred binding to the catalytic center of tyrosinase that for the binding of monophenols.

Kinetic studies of 9 on monophenolase activity

The effects of **9** (trans-3,4-difluorocinnamic acid) and kojic acid (positive control) on monophenolase activity of tyrosinase were evaluated in the oxidation reaction of L-tyrosine. The plots of optical density (OD) values at wavelength of 475 nm (OD_{475}) versus time were presented in Figure 2a-1 and b-1. Compared to the negative control (0 μM, without inhibitor), the steady-state rates (slope of the liner part of the curve) significantly decreased with the increasing concentration of **9** and kojic acid, indicating that **9** had a dose-dependent inhibitory effect on monophenolase activity as kojic acid. However, comparing the data of Figure 2a-2 with b-2, it was observed that **9** did not significantly prolong the lag time (kept at 1.67 ± 0.08 min) while kojic acid greatly prolonged the lag time from 1.71 ± 0.23 to 13.27 ± 0.89 min. This suggested that the compound had no inhibitory effects on the E_{met} type of tyrosinase (Chen *et al.* 2018), which can transform *o*-diphenol into *o*-quinone and be reduced to the E_{deoxy} type (Chang 2012). The plots of monophenolase activity versus the concentration of inhibitor were constructed, as shown in Figure 2a-3 and b-3, which were used to calculate the IC_{50} values that have been summarized in Table 2. The results demonstrated that **9** with IC_{50} value of 68.6 ± 4.2 μM was also an effective monophenolase inhibitor when compared to other cinnamic acid derivatives, despite that the inhibitory effect is appropriately 10 times lower than kojic acid with IC_{50} value of 7.76 ± 0.56 μM.

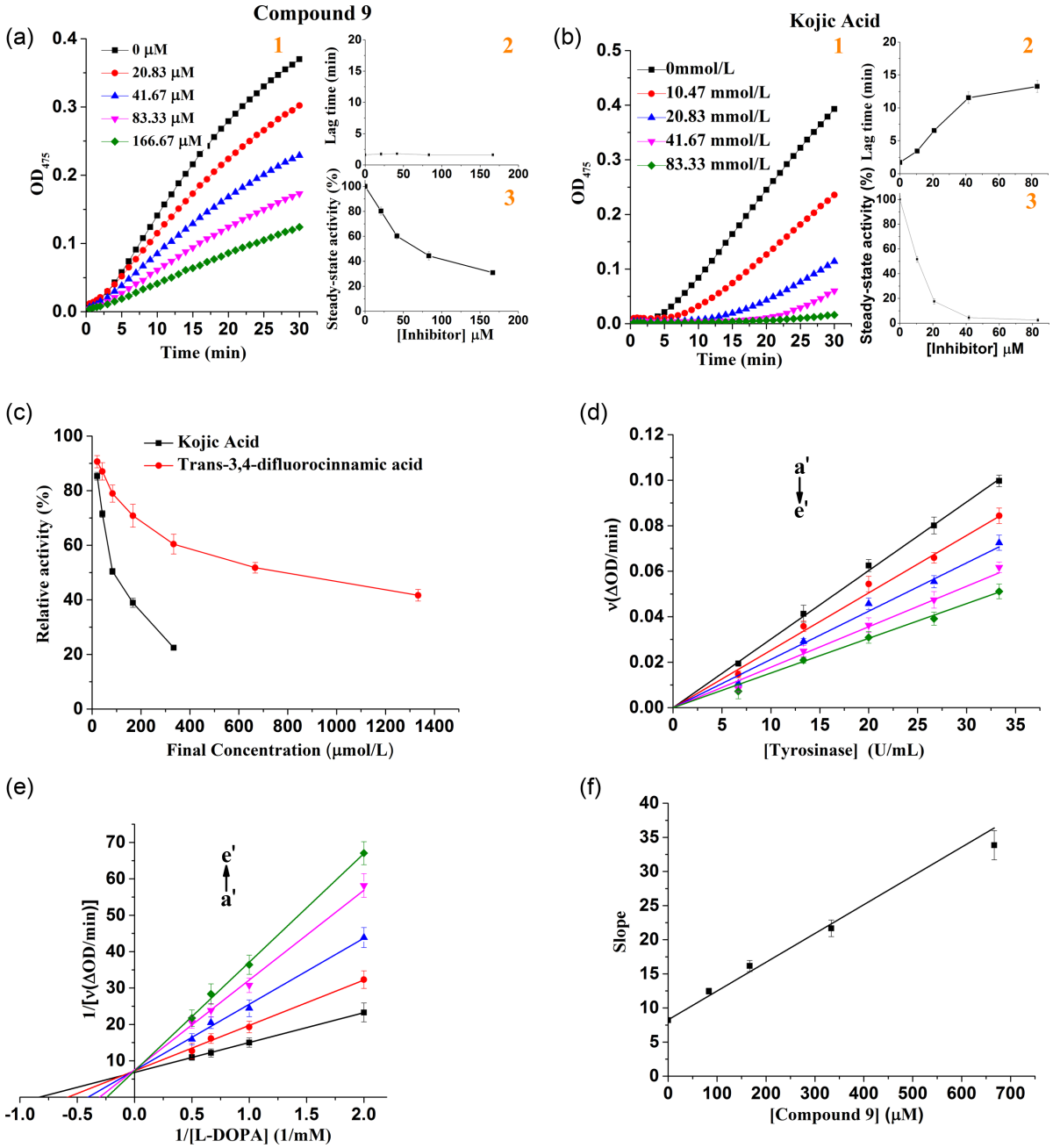

Figure 2. (a, b) Effects of different concentrations of **9** and kojic acid (positive control) on monophenolase activity (pH = 6.8, T = 305 K): progression of L-tyrosine oxidation by tyrosinase in the presence of different concentrations of **9** (a) and kojic acid (b). (c) Effects of **9** and kojic acid on diphenolase activity. The final concentrations of L-dopa and tyrosinase were 0.5 mM and 13.33 U/mL, respectively. (d) Plots of v versus [tyrosinase]. [L-dopa] = 0.5 mM, [**9**] = 0, 41.67, 166.67, 333.33, and 666.67 μM for curves (a′)-(e′), respectively. (e) Lineweaver–Burk plots. [tyrosinase] = 13.33 U/mL, [**9**] = 0, 83.33, 166.67, 333.33, and 666.67 μM for curves (a′)-(e′), respectively. (f) Secondary plot of slope versus [**9**]. The data points and bars represent the means ± SD (n = 3).

Inhibitory effect and mechanism of 9 on tyrosinase

The inhibitory effect of **9** on diphenolase activity was presented in Figure 2c along with kojic acid that was used as a positive control. Compound **9** showed the inhibitory effect in a concentration-dependent manner with the IC_{50} value of 0.78 ± 0.02 mM, which was 8 times higher than that of kojic acid (0.10 ± 0.01 mM). The inhibition mechanism of **9** on diphenolase activity was shown in Figure 2d, revealing that the enzyme activity decreased with the increasing concentration of the compound. Moreover, the plots of the reaction rates (v) versus the concentrations of tyrosinase in the presence of **9** with different concentrations gave a family of straight lines that all passed through the origin, suggesting that the inhibition of the enzyme by this compound was reversible (Chen *et al.* 2020a).

Inhibitory type of 9 on tyrosinase

The kinetic behavior of **9** against tyrosinase was then studied by using the double reciprocal Lineweaver–Burk plots. As shown in Figure 2e, the plots of 1/v versus 1/[S] gave a family of straight

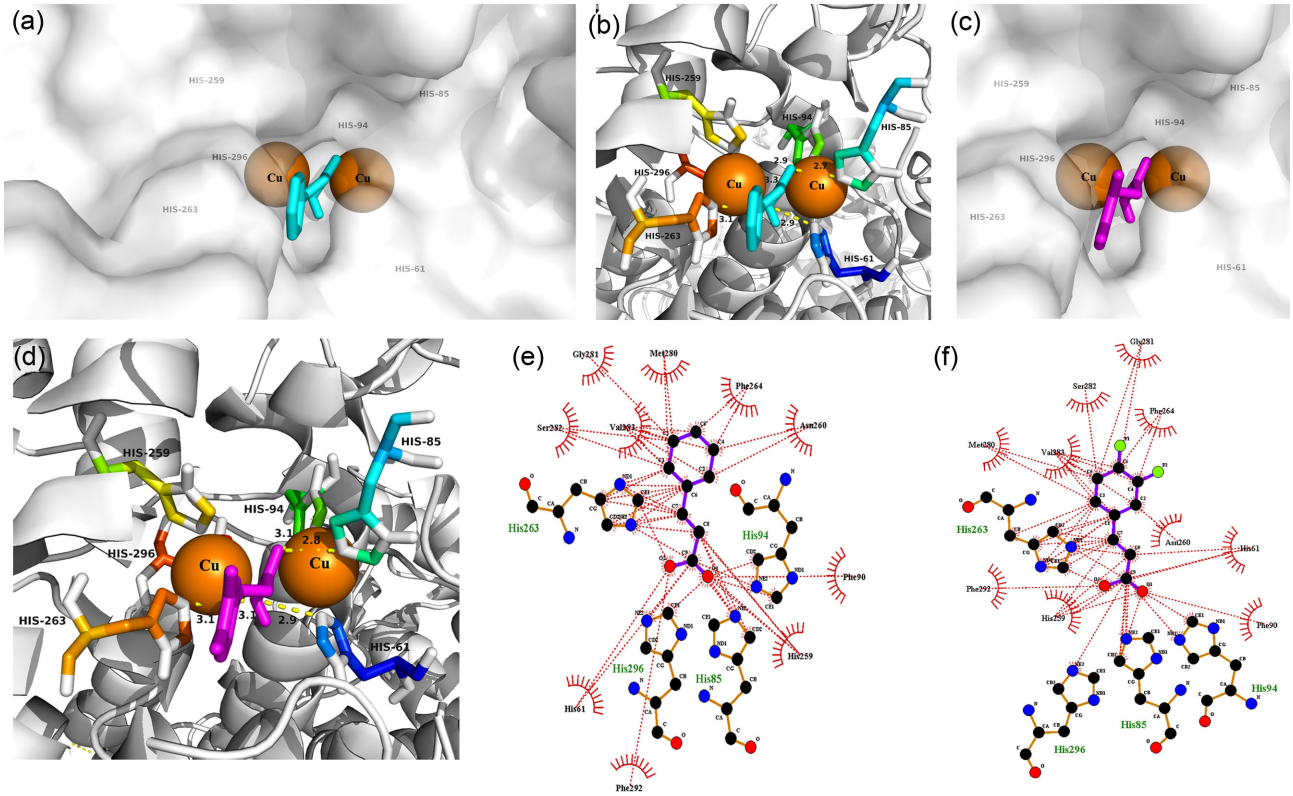

Figure 3. Docking simulations of binding of cinnamic acid and **9** with tyrosinase: (a) the surface structure of tyrosinase interacting with cinnamic acid (coarser stick structure in cyan); cinnamic acid is located in the catalytic center of the enzyme that is composed of 2 copper ions coordinating with 6 histidine residues. (b) The interaction details between cinnamic acid and tyrosinase, and the dashed yellow line represents the hydrogen bonds. (c) The surface structure of tyrosinase interacting with **9** (coarser stick structure in magenta). (d) The interaction details between **9** and tyrosinase. (e, f) The 2D ligand–protein interaction diagrams generated by Ligplot$^+$ describe the interactions of cinnamic acid and **9**, respectively, with the residues near the catalytic center of tyrosinase by hydrophobic interactions.

lines with a fixed intercept on the Y axis but with different slopes, indicating that the maximum reaction rate (υ_{max}) did not change and the values of K_m increased with the increasing concentrations of the inhibitor. Thus, it suggested that **9** was a competitive inhibitor that occupied the catalytic center of tyrosinase and competed with the substrate (L-dopa). The secondary plot of slope versus [**9**] was linear fitted (Figure 2f), suggesting that the compound had a single inhibition site or a single class of inhibition sites on tyrosinase (Zhang *et al.* 2017). The binding inhibition constant (K_i) of **9** was calculated to be 197 ± 11 μM. According to the competitive inhibition and the structure of the inhibitor, the structural similarity between **9** and substrate may be the main reason to lead to effective inhibition. Taken together, the results of kinetic and mechanism studies suggested that **9** could serve as a promising tyrosinase inhibitor.

Docking simulation of 9 binding to tyrosinase

The docking simulation was performed to further study the interaction between **9** and tyrosinase, and cinnamic acid was used as a reference compound. The conformations with the lowest docking energies (−9.10 and −9.56 kcal/mol for cinnamic acid and **9**, respectively) were taken as the best docking conformations, which were shown in Figure 3. The difference of docking energy between the 2 compounds suggested that **9** had higher affinity to tyrosinase than cinnamic acid, which was consisted with the results of *in vitro* assay. It was observed that both cinnamic acid (Figure 3a) and **9** (Figure 3c) were located in the pocket of the catalytic center of tyrosinase, which was composed of 2 copper ions cooperating with 6 histidine residues including His-61, His-85, His-94, His-263, His-259, and His-296 (Ismaya *et al.* 2011). More specifically, cinnamic acid (Figure 3b) and **9** (Figure 3d) interacted with the enzyme both by forming 5 hydrogen bonds with the same residues including His-61, His-85, His-94, His-263, and His-296.

Moreover, analysis of hydrophobic interaction by Ligplot$^+$ indicated that cinnamic acid (Figure 3e) and **9** (Figure 3f) surrounded by lots of residues. There were 14 residues involving in the hydrophobic interaction between cinnamic acid and tyrosinase, including Asn-260, Met-280, Gly-281, Ser-282, Val-283, Phe-90, Phe-264, Phe-292, His-61, His-85, His-94, His-259, His-263, and His-296. Interestingly, **9** surrounded by exactly the same residues as cinnamic acid, indicating that the 2 compounds shared the similar binding site with tyrosinase. More specifically, among these residues, there were 5 histidine residues including His-61, His-85, His-94, His-259, His-263, and His-296 that cooperated with copper ions to develop the catalytic center of tyrosinase involving in the hydrophobic interaction. Therefore, the strong hydrophobic interactions between **9** and tyrosinase may also play a key role in the formation of inhibitor-tyrosinase complex. These results suggested that **9** could compete with the substrate and inhibit the activity of tyrosinase by occupying the catalytic center of the enzyme, which were consisted with the results of our kinetic studies.

It is worth noting that the cinnamic acid and **9** showed the similar performance in docking simulation but the inhibition

Table 3. Effects of cinnamic acid and its derivatives against DPPH radical

Compound	EC_{50}[a] (μM)	Compound	EC_{50}[a] (μM)
1	>950	**10**	14.0 ± 1.6
2	>950	**11**	>950
3	>950	**12**	51.6 ± 2.5
4	>950	**13**	>950
5	>950	**14**	>950
6	>950	**15**	>950
7	>950	**16**	>950
8	>950	**17**	>950
9	>950	**18**	>950
Ascorbic acid	28.7 ± 2.3	Trolox	30.5 ± 1.8

[a]Values were obtained from the concentration–effect curves and expressed as means ± SD ($n = 3$).

mechanisms of them were different. The cinnamic acid has been reported to inhibit the tyrosinase in a noncompetitive manner whereas **9** inhibited in a competitive manner in this study (Shi *et al.* 2005). It was well known that competitive inhibitors share structural and/or charge features with the substrate but lack the ability to undergo catalysis and thereby preventing catalysis merely by occupying all or part of the substrate adsorptions pocket. Compound **9** shares the structural features with the substrate (L-dopa), because they both have substituents on 3- and 4-positions of the phenyl ring. Therefore, it may be due to the structural difference of **9** and cinnamic acid on the 3- and 4-positions that resulted in different inhibition mechanisms (Hu *et al.* 2014; Cui *et al.* 2017).

Antioxidant activities of cinnamic acid and its derivatives

The antioxidant activities of cinnamic acid and its derivatives were investigated by DPPH radical scavenging assay using ascorbic acid and Trolox as reference compounds. The EC_{50} (concentration required to obtain a 50% antioxidant effect) was utilized to express the antioxidant capacities of these compounds, which were summarized in Table 3. Among these tested compounds, only **10** (caffeic acid) and **12** (ferulic acid) exhibited considerable DPPH radical scavenging activities with EC_{50} values of 14.0 ± 1.6 and 51.6 ± 2.5 μM, respectively. Compared to ascorbic acid (28.7 ± 2.3 μM) and Trolox (30.5 ± 1.8 μM), it was found that the radical scavenging activity of caffeic acid was appropriately 2 times than that of the reference compounds. Compared to cinnamic acid, caffeic acid exhibited the excellent activity, which may be due to the 2 hydroxyl substituents on 3- and 4-positions of the phenyl ring. It was also observed that the substitution of 3-position hydroxyl with a methoxy group (**12**) resulted in a dramatic decrease of radical scavenging activity, which was consisted with the previous report (Barontini *et al.* 2014).

In addition, when the hydroxyl group of **12** was replaced with a methoxy group (**11**), it was found that the radical scavenging activity dropped dramatically. All these results suggested that hydroxyl groups played more positive roles in the antioxidant activity than methoxy groups, which may be due to that methoxy groups introduce unfavorable steric effects (Jeong *et al.* 2007) or prevent the formation of antioxidant o-quinone (Takahashi and Miyazawa 2010). Moreover, it was observed that the hydroxyl group on 4-position is essential to the activity, and methylation of this group diminished the scavenging activity, which was consisted with the previous report (Lu *et al.* 2006). However, in the support of the weak activity of **6** and **14**, we concluded that only 1 hydroxyl group on the phenyl ring was not enough to possess the radical scavenging activity, and the number of hydroxyl groups may be more important (Wu *et al.* 2012). Compared **12** and **13**, it was found that the position of hydroxyl and methoxy group also had a great impact on the activity. Interestingly, the addition of a methoxy group on the 3-position enhanced the activity when compared **12** with **6**. The analysis of SARs demonstrated the substituents on the phenyl ring except for hydroxyl group have no significant effect on the antioxidant activity.

In summary, the antityrosinase and antioxidant abilities of cinnamic acid and its derivates were evaluated, and the related SARs was analyzed. The results showed that most of these compounds exhibited the inhibitory effects on both monophenolase and diphenolase activity. Generally, the substituents on the phenyl ring resulted in the increment of inhibitory effects on monophenolase, while resulted in the reduction of inhibitory effects on diphenolase. This may be due to the independent binding sites in the catalytic center of tyrosinase for monophenols and diphenols, which led to the different SARs of the compounds inhibiting monophenolase and diphenolase. For monophenolase activity, it was found that the halogen substituent on 4-position of the phenyl ring enhanced the inhibitory effects on monophenolase activity as the increase of the electronegative values (F > Cl > Br). For diphenolase activity, compounds with substituents on the phenyl ring generally induced the reduction of the activity except for **9**. Specifically, **9** was first discovered as a reversible competitive inhibitor and had the most potency inhibitory effects on both monophenolase and diphenolase activity with IC_{50} values of 68.6 ± 4.2 μM and 0.78 ± 0.02 mM, respectively, which was further confirmed by the results of molecular docking study. From the results of antioxidant assay, it was found that only 1 hydroxyl group on the phenyl ring was not enough to possess the radical scavenging activity, and the number of hydroxyl groups may be more important. Moreover, the substituents except for hydroxyl and methoxy on the phenyl ring seemed to have no effect on the antioxidant capacity of cinnamic acid and its derivatives. The SARs demonstrated here will be helpful for the design and development of new cinnamic acid derivates as tyrosinase inhibitors and antioxidants with higher efficacy. Inspired by the excellent antityrosinase proprieties of **9**, we will further investigate whether it could have the potential to be applied in medicinal and agriculture industries.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contribution

J.C. and Y.Y. conceived the ideas, coordinated the overall project, and drafted the manuscript. M.R. and M.W. participated in the design of the study and conducted most of the experiments. S.L. and X.L. analyzed the data. All the authors reviewed the results and approved the final version of the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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