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Original article

Synthesis and biological evaluation of novel 4-hydroxybenzaldehyde derivatives as tyrosinase inhibitors

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ABSTRACT

A series of novel 4-hydroxybenzaldehyde derivatives were synthesized and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were investigated. Most of target compounds had more potent inhibitory activities than the parent compound 4-hydroxybenzaldehyde ($IC_{50} = 1.22$ mM). Interestingly, compound **3c** bearing a dimethoxyl phosphate was found to be the most potent inhibitor with IC_{50} value of 0.059 mM. The inhibition kinetics analyzed by Lineweaver–Burk plots revealed that compound **3c** was a non-competitive inhibitor ($K_I = 0.0368$ mM). In particular, compound **3c** showed no side effects at dose of 1600 mg/kg in mice. These results suggested that such compounds might be served as lead compounds for further designing new potential tyrosinase inhibitors.

1. Introduction

Tyrosinase is a multifunctional copper-containing enzyme widely distributed in microorganisms, plants and animals [1]. It is well known that tyrosinase can catalyzes two distinct reactions of melanin synthesis, the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) [2]. Previous reports confirmed that tyrosinase was involved, not only in melanizing in animals, but also in browning in plants. Recently, investigation demonstrated that various dermatological disorders, such as age spots and freckle, were caused by the accumulation of an excessive level of epidermal pigmentation [3]. Therefore, the control of tyrosinase activity is of great importance in preventing the synthesis of melanin in the browning of fruits and vegetables and the accumulation of an excessive level of epidermal pigmentation in animals.

Tyrosinase inhibitors have become increasingly important in medication [4], cosmetic industry [5] and food industry due to decreasing the excessive accumulation of pigmentation resulting from the enzyme action [6–10]. So far, numerous 4-substituted benzaldehyde derivatives and their analogues as potential tyrosinase inhibitors have been discovered from natural or synthetic sources, such as 4-hydroxybenzaldehyde [4], anisaldehyde [4], cuminaldehyde [11] and 4-methoxysalicylaldehyde [12] (Fig. 1). Unfortunately, most of 4-substituted benzaldehyde derivatives cannot be considered of practical use because of their lower activities or serious side effects. Therefore, it is still necessary to search and discover novel tyrosinase inhibitors with higher activities and lower side effects.

Previous literatures [4,12] reported that the 4-substituted benzaldehyde derivatives exhibited the tyrosinase inhibitory activity by the formation of a Schiff base between the aldehyde group and a primary amino group of the enzyme, and the inductive effect of the electron-donating group at 4-position are necessary for the actions [11]. However, the inductive effect cannot be the only explanation for the binding stability of the enzyme and the inhibitor [1]. In addition, it is well known that phosphates and amino acids are involved in the metabolism of organism and exhibit various biochemical and pharmacological functions. More recently, our groups reported that condensation products of 4-formylphenoxyacetic acid and the appropriate amino acid methyl esters displayed potent acetylcholinesterase inhibitory activities [13].

Taking advantage of previously developed structure–activitiy relationships (SARs) of 4-substituted benzaldehydes as tyrosinase inhibitors, in the present investigation, a series of novel 4-hydroxybenzaldehyde derivatives were synthesized and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were evaluated. Meanwhile, the inhibition mechanism and

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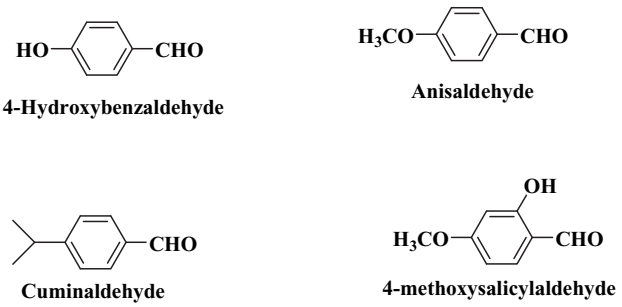
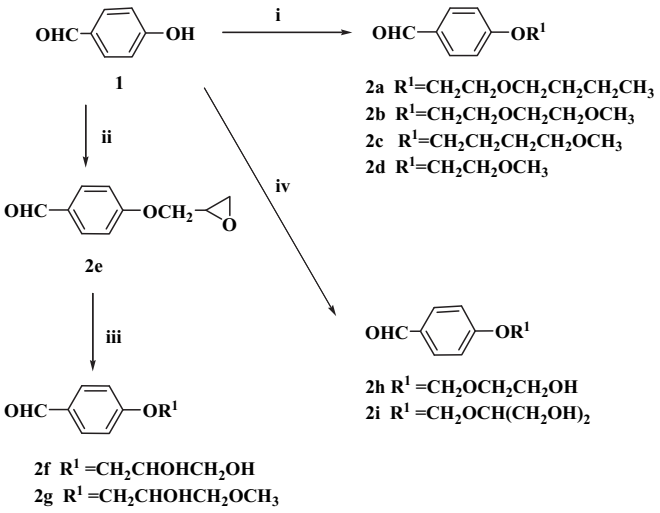


Fig. 1. Chemical structures of known tyrosinase inhibitors.



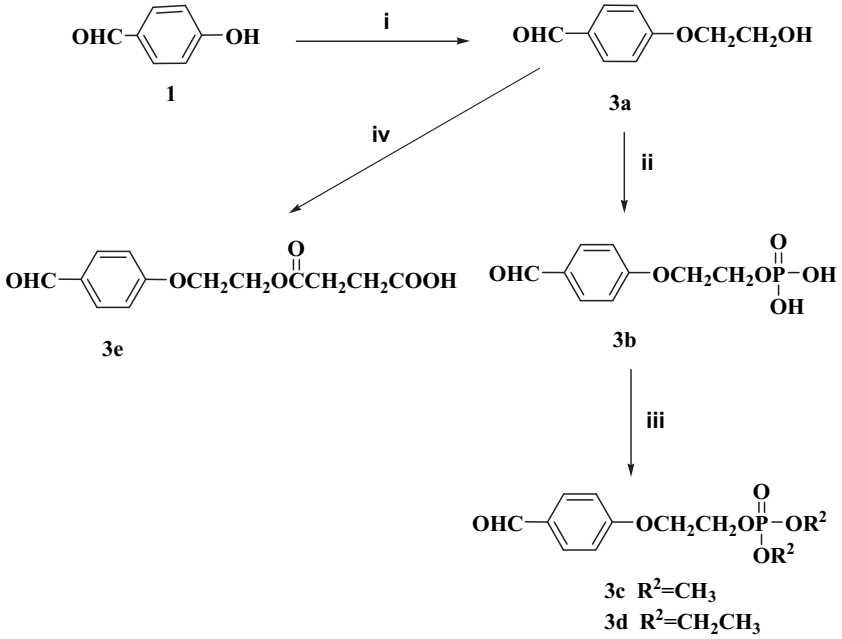
Scheme 1. Synthesis of compounds **2a**–**i**. Reagents and conditions: (i) 2-butoxyethanol or 2-(2-methoxyethoxy)ethanol or 4-methoxybutan-1-ol or 2-methoxyethanol, NaOH, TsCl, 0 °C, 5 h; K_2CO_3, THF, reflux, 16 h (ii) epichlorohydrin, 1,4-dioxane, NaOH, H_2O, reflux, 2 h (iii) NaOH, H_2O, reflux, 3 h; methanol, sodium methoxide, reflux, 24 h (iv) 2-oxo-1,4-butanediol diacetate ester or 2-[(propionyloxy)methyl-hydrogen]-1,3-propanediol diacetate, toluene, 4-methylbenzenesulfonic acid, 100 °C, 0.5 h, then reflux, 10 h; methanol, sodium methoxide, rt, 1 h.

preliminary acute toxicity of the most potent compound were also investigated. The purpose of this study was to investigate effects of substituents at postion-4 of benzene on tyrosinase inhibitory activity, with the ultimate aim of developing novel tyrosinase inhibitors with potent activities, together with lower side effects.

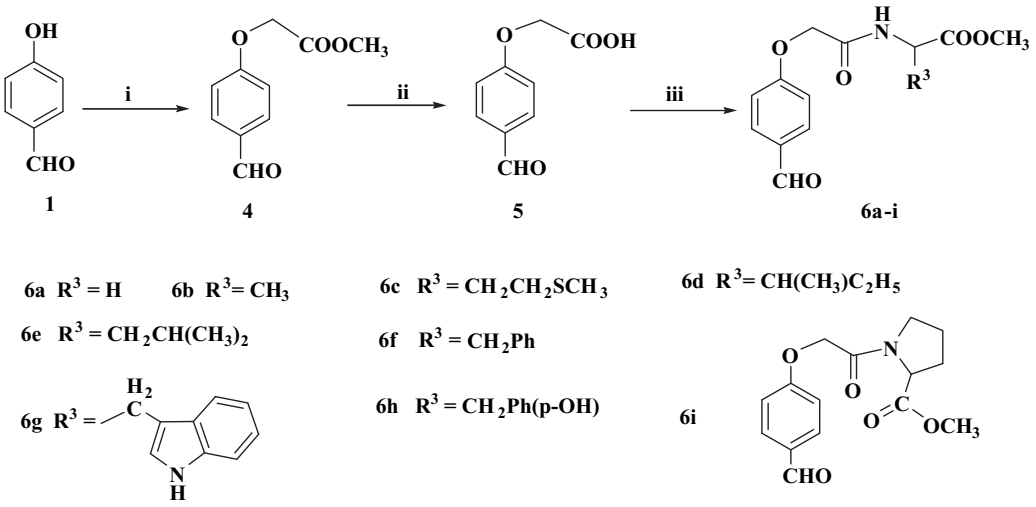
2. Chemistry

The synthesis of 4-hydroxybenzaldehyde derivatives was summarized in Scheme 1–3. The reaction of 4-hydroxybenzaldehyde with 2-chloroethanol and epichlorohydrin, respectively, in 1,4-dioxane and water gave the expected derivatives **3a** and **2e** in good yield [14,15]. The intermediate 2-butoxyethyl-4-methylbenzene sulfonate was prepared from the commercially available 2-butoxyethanol and TsCl according to already published methods [16]. Then, 2-butoxyethyl-4-methylbenzene sulfonate was reacted with 4-hydroxybenzaldehyde in anhydrous THF in the presence of anhydrous potassium carbonate to afford the derivative **2a**. Following the similar procedure, compounds **2b**–**d** could be carried out smoothly in good yield. Compound **2e** was hydrolyzed in sodium hydroxide solution to afford compound **2f**. Esterification of compound **2e** with sodium methoxide in methanol provided compound **2g**. The intermediate 2-(4-formyl-phenoxy)-methoxyethyl acetate was prepared from the starting materials 2-oxo-1,4-butanediol diacetate ester, 4-methylbenzenesulfonic acid and 4-hydroxybenzaldehyde according to the usual methods [17,18], followed by hydrolyzation in NaOH solution to provide the target compound **2h**. Compound **2i** was given according to the same procedure described for compound **2h** (Scheme 1).

Compound **3a** reacted with phosphorous oxychloride in anhydrous dichloromethane, followed by hydrolyzation in sodium hydroxide solution to provide compound **3b**, and followed by esterification with anhydrous methanol in the presence of sodium methoxide and with anhydrous ethanol in the presence of sodium ethoxide to provide compound **3c** and **3d**, respectively. Esterification of compound **2a** with succinic anhydride in anhydrous dichloromethane gave compound **3e** (Scheme 2).



Scheme 2. Synthesis of compounds **3a**–**e**. Reagents and conditions: (i) 2-chloroethanol, 1,4-dioxane, NaOH, H_2O, reflux, 2 h (ii) Et_3N, CH_2Cl_2, $POCl_3$, 0 °C, 1.5 h; NaOH, rt, 5 h (iii) methanol/sodium methoxide or ethanol/sodium ethoxide, reflux, 2 h (iv) Et_3N, CH_2Cl_2, succinic anhydride, 100 °C, 0.5 h; DMAP, reflux, 4 h.



Scheme 3. Synthesis of compounds **6a–i**. Reagents and conditions: (i) $ClCH_2COOCH_3$, K_2CO_3, acetone, 60 °C, 5 h (ii) NaOH, H_2O; H^+, H_2O. (iii) DCC, DAMP, $CHCl_3$, rt, overnight.

The reaction of compound **1** with methyl chloroacetate gave compound **4**, followed by hydrolyzation in sodium hydroxide to provide 4-formylphenoxyacetic acid **5**. The condensation of various amino acid methyl esters with compound **5** in the presence of N,N′-dicyclohexyl-carbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) produced compound **6a–i** [13] (Scheme 3).

3. Results and discussion

3.1. Inhibitory effects on the diphenolase activity of mushroom tyrosinase

Taking compound **2a–i**, **3a–e** and **6a–i** as the effectors, we investigated the inhibitory effects of 4-hydroxybenzaldehyde derivatives on the diphenolase activity of mushroom tyrosinase for the oxidation of L-DOPA. Fig. 2 showed that the remaining enzyme activity rapidly decreased with the increasing concentrations of compound **3c**. The IC_{50} value of all compounds investigated was summarized in Table 1.

As shown in Table 1, all compounds exhibited tyrosinase inhibitory effects with IC_{50} values ranged from 0.059 to 3.26 mM.

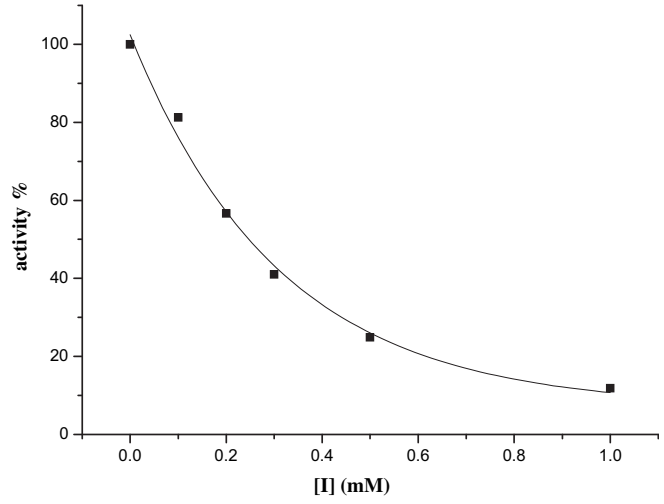


Fig. 2. Effect of compound **3c** on the diphenolase activity of mushroom tyrosinase for the catalysis of L-DOPA at 25 °C.

And compounds **2b–e**, **2g–f**, **3b–e**, **6a–d** and **6f–i** displayed inhibitory effects on the diphenolase activity of mushroom tyrosinase in the sub-millimolar ranges. Interestingly, compound **3c** bearing a dimethoxyl phosphate substituent was found to be the most potent inhibitor with IC_{50} value of 0.059 mM. Replacement of methoxy group of compound **3c** with ethoxy group led to a dramatic decline in tyrosinase inhibitory activity (compound **3d**, $IC_{50} = 0.55$ mM). This might be due to molecular dimension of the ethoxy substitution to impede this molecule to interact well with the enzyme. In addition, compound **3b** ($IC_{50} = 0.82$ mM) bearing a free phosphate group and compound **3e** ($IC_{50} = 0.62$ mM) having a free carboxyl substituent showed moderate tyrosinase inhibitory activities. These results suggested that introducing appropriate hydrophobic subunits into position-4 of benzaldehyde might be facilitated their inhibitory effects on the diphenolase activity of mushroom tyrosinase, and dimethoxyl phosphate group represented the most optimal structure for these compounds to exhibit remarkable tyrosinase inhibitory effects.

Compound **3a** bearing a hydroxyethoxyl group at position-4 of phenyl ring exhibited weak tyrosinase inhibitory activity with IC_{50} value of 2.55 mM. Interestingly, replacement of the hydroxyl group of compound **3a** with methoxy (compound **2d**, $IC_{50} = 0.66$ mM) and methoxyethoxy (compound **2b**, $IC_{50} = 0.9$ mM), respectively,

Table 1
Inhibitory effects on mushroom tyrosinase of 4-substituted benzaldehyde derivatives.

Compound	IC_{50}(mmol/L)[a]	Compound	IC_{50}(mmol/L)[a]
2a	3.26	**3e**	0.62
2b	0.90	**6a**	0.17
2c	0.62	**6b**	0.16
2d	0.66	**6c**	0.40
2e	0.59	**6d**	0.46
2f	1.26	**6e**	1.65
2g	0.75	**6f**	0.48
2h	0.37	**6g**	0.11
2i	0.27	**6h**	0.22
3a	2.55	**6i**	0.76
3b	0.82	**1**	1.22[b]
3c	0.059	4-methoxyinnamic acid	0.41[c]
3d	0.55		

[a] Values were determined from logarithmic concentration–inhibition curves (at least eight points) and are given as means of three experiments.
[b] Values in the literature [4] is 1.20 mM.
[c] Values in the literature [20] is 0.42 mM.

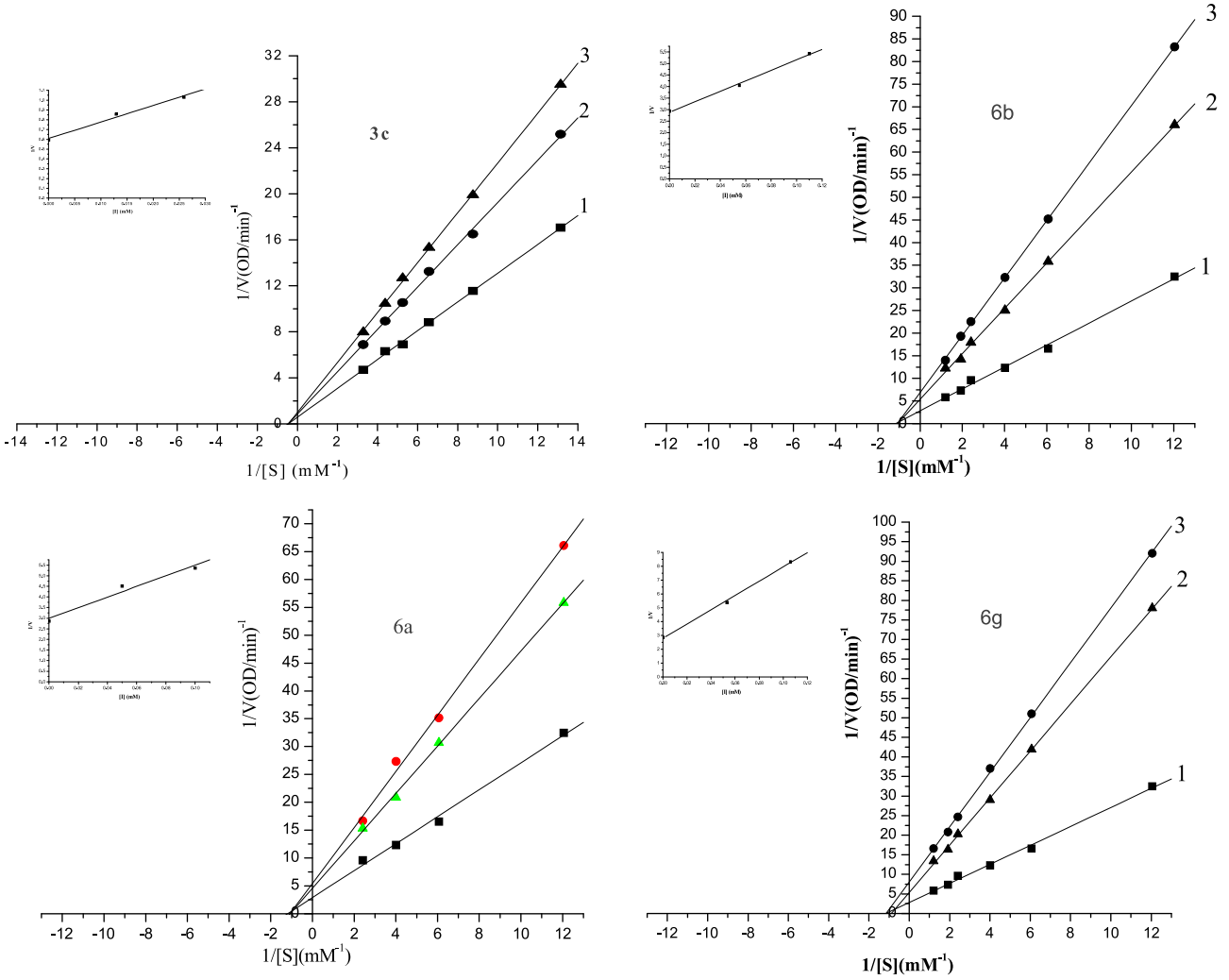


Fig. 3. Lineweaver–Burk plots for inhibition of selected compounds **3c**, **6a**, **6b** and **6g** on mushroom tyrosinase for the catalysis of L-DOPA. Concentrations of **3c**, **6a**, **6b** and **6g** for curves 1–3 were 0, 12.94 μM, 25.88 μM; 0, 0.05 mM, 0.10 mM; 0, 0.055 mM, 0.11 mM; 0, 0.0503 mM, 0.106 mM, respectively. The inset represents the secondary plot of $1/V_{max}$ versus concentration of **3c**, **6a**, **6b** and **6g**, respectively, to determine the inhibition constants (K_I).

led to a dramatic increase in inhibitory activities. Whereas compound **2a** ($IC_{50} = 3.26$ mM) having a butoxyethoxy substituent was 4.9 times less active than compound **2d** ($IC_{50} = 0.66$ mM). These results indicated that the terminal methoxy group contributed to tyrosinase inhibitory activities, but the elongation of the alkyl chain might retard the binding of inhibitors with the active site of tyrosinase, leading to a decrease of tyrosinase inhibition activity.

Compound **2e** ($IC_{50} = 0.59$ mM) bearing ethylene oxide moiety in the side chain showed potent tyrosinase inhibitory activities, whereas its opened-ring congener **2f** ($IC_{50} = 1.26$ mM) exhibited weaker inhibitory effect. Interestingly, replacement of terminal hydroxyl group of compound **2f** with a methoxy substituent to give compound **2g** ($IC_{50} = 0.75$ mM) demonstrated more potent tyrosinase inhibitory activities. These results suggested that the opened-ring of ethylene oxide might be detrimental to tyrosinase inhibitory activity.

Unlike compound **3a** having a hydroxyethoxyl group at position-4 of phenyl ring, compound **2h** ($IC_{50} = 0.37$ mM) bearing an additional oxygen in the side chain attached to phenyl ring exhibited potent tyrosinase inhibitory activity. Similarly, the inhibitory activity of compound **2i** ($IC_{50} = 0.27$ mM) was 4.5 times than that of compound **2f** ($IC_{50} = 1.26$ mM). These resulted indicated that the additional oxygen in the side chain might play an important role in determining their inhibitory effects on the diphenlosase activity of mushroom tyrosinase.

Our previous report [13] described that the condensation products of 4-formylphenxoyacetic acid and various amino acid methyl esters exhibited potent acetylcholinesterase inhibitory activities. In this investigation, these compounds were subjected for tyrosinase inhibition assays. As shown in Table 1, all compounds (except **6e**) exhibited more potent inhibitory activities than the parent compound 4-hydroxybenzadehyde ($IC_{50} = 1.22$ mM). Especially, compounds **6a**, **6b** and **6g** displayed significant tyrosinase inhibitory activities with IC_{50} value of 0.17, 0.16 and 0.11 mM, respectively. These results suggested that the introduction of appropriate amino acid moieties into position-4 of benzaldehyde might be enhanced their inhibitory effects on the diphenolase activities of mushroom tyrosinase, and L-glycine (**6a**), L-alanine (**6b**) and L-tryptophan (**6g**) was more favorable.

3.2. Inhibition mechanism of the selected compounds on mushroom tyrosinase

The inhibitory mechanism of the selected compounds **3c**, **6a**, **6b** and **6g** on mushroom tyrosinase for the oxidation of L-DOPA was

Table 2
Kinetics and inhibition constants of compounds **3c**, **6a**, **6b** and **6g** on the activity of mushroom tyrosinase.

Compound	Inhibition type	Inhibition constant (K_I) (mM)
3c	Non-competitive	0.0368
6a	Non-competitive	0.117
6b	Non-competitive	0.125
6g	Non-competitive	0.054

determined from Lineweaver–Burk double reciprocal plots. Fig. 3 showed the double-reciprocal plots of the enzyme inhibited by the above-mentioned compounds. The results displayed that the plots of $1/V$ versus $1/[S]$ gave three straight lines with different slopes, but intersected on the horizontal axis. Accompanying the increase of the concentration of compound, the value of V_{max} descended but the values of K_m remained the same, which suggested that these compounds was a non-competitive inhibitor of the tyrosinase. The behaviour indicated that these compounds could bind with both the free enzyme and the enzyme–substrate complex, and the equilibrium constants are the same [19]. The inhibition constants for compounds **3c**, **6a**, **6b** and **6g** binding with the free enzyme (K_I) were determined by the plot of the values of intercept versus the concentration of the corresponding compound as shown in Fig. 3. The values obtained are summarized in Table 2. As shown in Table 2, the inhibition constant (K_I) of compound **3c** was less than that of compounds **6a**, **6b** and **6g**, suggesting that compound **3c** had most potent inhibitory effect.

The reason for the exceptionally potent inhibition activity of compound **3c** might be that, to a certain extent, intermolecular hydrogen bond formed between the dimethoxyl phosphate group of compound **3c** and the sulfhydryl, amino, carboxyl or hydroxyl in tyrosinase, and the interaction increased the chance for aldehyde group of compound **3c** to form Schiff base with amino group locating in the outside of enzyme active center [20]. Undoubtedly, the more the numbers of hydrogen bond formed between compound **3c** and tyrosinase, the tighter and the more stable the interaction between **3c** and tyrosinase. This assumption is consistent with the deduction in the previous report that the hydrogen-bonding interactions could stabilize the *oxy*-form of *Streptomyces glaucescens* tyrosinase [21]. However, the conclusive interpretation remains to be clarified since the structure of tyrosinase used for this study has not yet been understood completely.

3.3. Acute toxicity of compound *3c* in mice

Compound **3c**, the most potent inhibitor of this investigation, was selected to evaluate acute toxicity in mice. Clinical symptom was measured for 20 days after the oral single gavage administration of 1600 mg/kg. The results showed that all of the mice after administration at dose of 1600 mg/kg body weight/day did not show any mortality, and autopsy of the animals at the end of the experimental period (20 days) revealed no apparent changes in any organs. The results indicated that the compound **3c** was safe at dose of 1600 mg/kg in mice.

4. Conclusion

The present investigation reported the effects of 4-hydroxybenzaldehyde derivatives on the diphenolase activity of mushroom tyrosinase for the oxidation of L-DOPA. The results demonstrated that most of target compounds had more potent inhibitory activities than the parent compound 4-hydroxybenzaldehyde. Preliminary SARs analysis indicated that: (1) the numbers of oxygen atoms contained in the chain attached position-4 of benzaldehyde affected tyrosinase activity; (2) the elongation of the alkyl chain might retard the binding of inhibitors with the active site of tyrosinase, leading to a decrease of tyrosinase inhibition activities; (3) the molecular symmetry might be play a very important role in determining enzyme activities; (4) the introduction of the appropriate amino acid moieties such as L-glycine, L-alanine, L-tryptophan enhanced inhibitory activities. Interestingly, compound **3c** bearing a dimethoxyl phosphate was found to be the most potent inhibitor with IC_{50} value of 0.059 mM. The inhibition kinetics analyzed by Lineweaver–Burk plots revealed that compound **3c** was a non-competitive inhibitor ($K_I = 0.0368$ mM). Safety is a primary consideration for tyrosinase inhibitors, especially for those materials used in food and cosmetic products. It is worth noting that compound **3c** did not showed any side effects at dose of 1600 mg/kg in mice. All these data suggested that such compounds might serve as candidates for the treatment of tyrosinase based disorders and as lead compounds for further designing new potential tyrosinase inhibitors.

5. Experimental protocols

5.1. Reagents and general procedures

Melting points were determined on a WRS-1B digital instrument without correction. ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury-Plus 300 NMR instrument (^1H 300 MHz; ^{13}C 75 MHz) in either $CDCl_3$ or DMSO-d_6. Abbreviations for data quoted are s, singlet; br s, broad singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet. Mass spectra were recorded on a Thermo Finnigan LCQ DECAXP ion trap mass spectrometer or VS ZAB-HS spectrometer. IR spectra were recorded as potassium bromide pellets on a Bruker Equinox 55 FT/IR spectrometer. Elemental analyses (C, H, N) were carried out on an Elementary Vario EL series elemental analyzer and the results were within ±0.4%. Thin-layer chromatographies were done on pre-coated silica gel 60 F254 plates (Merck).

Tyrosinase and L-3, 4-dhydroxyphenylalanine (L-DOPA) were purchased from Sigma–Aldrich Chemical Co. Other chemicals were purchased from commercial suppliers and were dried and purified when necessary. The water used was re-distillated and ion-free. Compounds **4**, **5** and **6a–i** were synthesized, purified and characterized as previously described [13].

5.2. Synthesis

5.2.1. 4-(2-Butoxyethoxy)benzaldehyde (*2a*)

A mixture of 2-butoxyethanol (6.0 g, 50 mmol), sodium hydroxide (4.0 g, 100 mmol) and water (10 ml) was cooled to 0 °C, and then TsCl (20.96 g, 11 mmol) in anhydrous THF was added dropwise and stirred for 5 h. The resulting mixture was treated with sodium hydroxide (1.6 g, 40 mmol) and extracted with chloroform. The organic layer was dried over anhydrous magnesium sulfate, filtered and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 1:5) to afford 2-butoxyethyl-4-methylbenzene sulfonate (11.88 g, 92.0%) as a liquid. ^1H NMR ($CDCl_3$, 300 MHz) δ: 7.79 (d, $J = 8.4$ Hz, 2H, ArH), 7.32 (d, $J = 8.4$ Hz, 2H, ArH), 4.60 (t, $J = 5.3$ Hz, 2H, CH_2), 4.15 (t, $J = 5.3$ Hz, 2H, CH_2), 3.40 (t, $J = 4.6$ Hz, 2H, CH_2), 2.44 (s, 3H, CH_3), 1.46 (m, 2H, CH_2), 1.31 (m, 2H, CH_2), 0.89 (t, $J = 4.6$ Hz, 3H, CH_3).

A mixture of 2-butoxyethyl-4-methylbenzene sulfonate (2.74 g, 10 mmol), 4-hydroxybenzaldehyde (1.22 g, 10 mmol), anhydrous potassium carbonate (2.07 g, 15 mmol) and anhydrous THF (25 ml) was refluxed for 16 h. The reaction mixture was poured into ice water and extracted with chloroform. The organic layer was dried

over anhydrous magnesium sulfate, filtered and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate:petroleum ether = 1:5) to give pale yellow oil (1.63 g, 74%). ESI-MS: *m/z* 223 (M + 1). IR (KBr, cm^{-1}) *ν*: 2958(C–H), 1693 (CHO), 1601 (Ph), 1509 (Ph), 1259 (C–O), 1161 (C–O), 1126 (C–O), 833 (Ph); ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.88 (s, 1H, CHO), 7.82(d, *J* = 8.3 Hz, 2H, ArH), 7.02 (d, *J* = 8.3 Hz, 2H, ArH), 4.20 (t, *J* = 5.5 Hz, 2H, CH_2), 3.80 (t, *J* = 5.5 Hz, 2H, CH_2), 3.54 (t, *J* = 4.8 Hz, 2H, CH_2), 1.59 (m, 2H, CH_2), 1.35 (m, 2H, CH_2), 0.93 (t, *J* = 4.5 Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) *δ*: 190.4, 163.6, 131.6, 129.7, 114.6, 71.2, 68.7, 67.6, 31.5, 19.1, 13.8; Anal. Calcd for $C_{13}H_{18}O_3$: C, 70.27; H, 8.11; found: C, 70.09; H, 8.34.

*5.2.2. 4-[2-(2-Methoxyethoxy)ethoxy]benzaldehyde (**2b**)*

Compound **2b** (1.62 g, 73%) was obtained as pale yellow solid by the same procedure described for **2a** from 4-hydroxybenzaldehyde (1.22 g, 10 mmol) and 2-(2-methoxyethoxy)ethanol (6.0 g, 50 mmol). Mp 44–45 °C. ESI-MS: *m/z* 225 (M + 1). IR (KBr, cm^{-1}) *ν*: 2891, 1689, 1601, 1509, 1129, 1104, 1034, 836; ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.89 (s, 1H, CHO), 7.81 (d, *J* = 8.7 Hz, 2H, ArH), 7.02 (d, *J* = 8.7 Hz, 2H, ArH), 4.23 (t, *J* = 4.8 Hz, 2H, CH_2), 3.90 (t, *J* = 4.8 Hz, 2H, CH_2), 3.73 (dd, *J* = 2.0 Hz, 4.8 Hz, 2H, CH_2), 3.59 (dd, 2H, *J* = 2.0 Hz, 4.8 Hz, CH_2), 3.34 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) *δ*: 190.4, 163.2, 131.6, 129.7, 114.6, 71.7, 70.6, 69.3, 67.2, 58.9; Anal. Calcd for $C_{12}H_{16}O_4$: C, 64.27, H, 7.19; found: C, 64.35; H, 7.12.

*5.2.3. 4-(4-Methoxybutoxy)benzaldehyde (**2c**)*

Compound **2c** (1.43 g, 69.0%) was obtained as yellow oil by the same procedure described for **2a** from 4-hydroxybenzaldehyde (1.22 g, 10 mmol) and 4-methoxybutan-1-ol (5.21 g, 50 mmol). ESI-MS: *m/z* 209 (M + 1). IR (KBr, cm^{-1}) *ν*: 2950, 1691, 1600, 1509, 1253, 1160, 1125, 834; ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.90 (s, 1H, CHO), 7.76 (d, *J* = 8.4 Hz, 2H, ArH), 7.01(d, *J* = 8.4 Hz, 2H, ArH), 4.15 (t, *J* = 4.5 Hz, 2H, CH_2), 3.76 (t, *J* = 6.3 Hz, CH_2, 2H,), 3.37 (s, 3H, OCH_3), 1.73 (m, 2H, CH_2), 1.65 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) *δ*: 190.4, 163.9, 131.7, 129.6, 114.5, 72.1, 68.1, 58.4, 26.1, 25.9; Anal. Calcd for $C_{12}H_{16}O_3$: C, 69.21; H, 7.74; found: C, 69.12; H, 7.88.

*5.2.4. 4-(2-Methoxyethoxy)benzaldehyde (**2d**)*

Compound **2d** (1.48 g, 82%) was obtained as yellow oil by the same procedure described for **2a** using 4-hydroxybenzaldehyde (1.22 g, 10 mmol) and 2-methoxyethanol (3.83 g, 50 mmol) as starting materials. EI-MS: *m/z* 180 (M + 1). IR (KBr, cm^{-1}) *ν*: 2925, 1690, 1600, 1508, 1258, 1159, 1124, 833; ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.90 (s, 1H, CHO), 7.79 (d, *J* = 8.4 Hz, 2H, ArH), 7.02 (d, *J* = 8.4 Hz, 2H, ArH), 4.19 (t, *J* = 5.1 Hz, 2H, CH_2), 3.79 (t, *J* = 5.1 Hz, 2H, CH_2), 3.47 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) *δ*: 190.3, 163.4, 131.5, 129.7, 114.5, 70.4, 67.4, 58.9; Anal. Calcd for $C_{10}H_{12}O_3$: C, 66.67; H, 6.67; found: C, 66.42; H, 6.85.

*5.2.5. 4-(2-Oxiranylmethoxy)benzaldehyde (**2e**)*

A mixture of 4-hydroxybenzaldehyde (1.22 g, 10 mmol), epichlorohydrin (3 ml), 1,4-dioxane (15 ml), water (5 ml) and sodium hydroxide (4.4 g, 110 mmol) was refluxed for 2 h. The solvent was removed under reduced pressure and the residue was extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 1:4) to afford compound **2e** (1.49 g, 83%) as a yellow oil. ESI-MS: *m/z* 179 (M + 1). ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.86 (s, 1H, CHO), 7.81 (d, *J* = 8.8 Hz, 2H, ArH), 7.01 (d, *J* = 8.8 Hz, 2H, ArH), 4.34 (m, 1H, CH_2), 4.01 (m, 1H, CH_2), 3.38 (m, 1H, CH); 2.93 (m, 1H, CH_2), 2.71 (m, 1H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) *δ*: 190.3, 162.9, 131.5, 129.8, 114.5, 68.7, 49.6, 44.2; Anal. Calcd for $C_{10}H_{10}O_3$: C, 67.41; H, 5.66; found: C, 67.68; H, 6.45.

*5.2.6. 4-(2,3-Dihydroxypropoxy)benzaldehyde (**2f**)*

A mixture of compound **2e** (1.78 g, 10 mmol) and 1 M NaOH solution (10 mL) was refluxed for 3 h. The solvent was removed under reduced pressure and the residue was extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 1:4) to afford compound **2f** (1.17 g, 57%) as yellow oil. ESI-MS: *m/z* 195 *m/z* (M − 1). IR (KBr, cm^{-1}) *ν*: 3370, 1684, 1600, 150, 1309, 1164, 1112, 1034, 839; ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.82 (s, 1H, CHO), 7.78 (d, *J* = 8.8 Hz, 2H, ArH), 6.97 (d, *J* = 8.8 Hz, 2H, ArH), 4.26 (m, 1H, CH), 4.19 (m, 2H, CH_2), 3.78 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) *δ*: 190.3, 163.0, 131.6, 130.0, 114.6, 72.5, 70.2, 68.8; Anal. Calcd for $C_{10}H_{12}O_4$: C, 61.22; H, 6.12; found: C, 62.45; H, 5.98.

*5.2.7. 4-(2-Hydroxy-3-methoxypropoxy)benzaldehyde (**2g**)*

A mixture of compound **2e** (1.78 g, 10 mmol) and sodium methoxide in methanol (0.2 M, 20 ml) was refluxed for 24 h. The solvent was removed under reduced pressure and the residue was extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 1:4) to afford compound **2g** (1.17 g, 57%) as pale yellow oil. ESI-MS: *m/z* 211 (M + 1). ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.87 (s, 1H, CHO), 7.82 (d, *J* = 8.6 Hz, 2H, ArH), 7.02 (d, *J* = 8.6 Hz, 2H, ArH), 4.18 (m, 1H, CH), 4.11 (m, 2H, CH_2), 3.58 (m, 2H, CH_2); 3.43 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) *δ*: 190.5, 163.3, 131.8, 130.0, 114.6, 73.0, 68.9, 68.6, 59.1; Anal. Calcd for $C_{11}H_{11}O_4$: C, 62.85; H, 6.71; found: C, 63.11; H, 6.58.

*5.2.8. 4-(2-Hydroxyethoxymethoxyl)benzaldehyde (**2h**)*

2-Oxo-1,4-butanediol diacetate ester (4.4 g, 25 mmol) in toluene (50 ml) was added to 4-methylbenzenesulfonic acid (0.35 g, 1.5 mmol). The mixture was stirred at 100 °C for 0.5 h. Then, 4-hydroxybenzaldehyde (3.05 g, 25 mmol) was added and the solution was refluxed for 10 h. The resulting mixture was evaporated under reduced pressure to remove the toluene and extracted with chloroform. The organic layer was washed with water and sodium hydroxide (1 M), then dried over anhydrous magnesium sulfate, filtered and evaporated. The crude product was purified by silica gel column chromatography, and the intermediate 2-(4-formylphenoxy)methoxyethyl acetate (3.37 g, 57%) was obtained as colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.88 (s, 1H, CHO), 7.82 (d, *J* = 8.7 Hz, 2H, ArH), 7.15 (d, *J* = 8.7 Hz, 2H, ArH), 5.32 (s, 2H, CH_2), 4.23 (m, 2H, CH_2), 3.89 (m, 2H, CH_2), 1.97 (s, 3H, CH_3).

Then, the intermediate 2-(4-formylphenoxy)methoxyethyl acetate (2.0 g, 8.4 mmol) was added to a solution of sodium methoxide-methanol (0.2 M, 20 ml). The reaction mixture was stirred for 1 h at room temperature, and methanol was evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (ethyl acetate:petroleum ether = 3:7). Compound **2h** (1.60 g, 97.0%) was obtained as a liquid. ESI-MS: *m/z* 195 (M − 1). IR (KBr, cm^{-1}) *ν*: 3420, 2925, 1686, 1601, 1508, 1231, 1162, 1068, 983, 835; ^{1}H NMR ($CDCl_3$, 300 MHz) *δ*: 9.87 (s, 1H, CHO), 7.83 (d, *J* = 8.7 Hz, 2H, ArH), 7.14 (d, *J* = 8.7 Hz, 2H, ArH), 5.35 (s, 2H, CH_2), 3.83 (m, 2H, CH_2), 3.76 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) *δ*: 190.8, 161.8, 131.7, 130.5, 116.1, 93.0, 70.5, 61.5; Anal. Calcd for $C_{10}H_{12}O_4$: C, 61.22; H, 6.12; found: C, 61.08; H, 6.41.

*5.2.9. 2-(4-Formylphenoxy)methoxypropane-1,3-diol (**2i**)*

Compound **2i** (1.17 g, 26%) was obtained as colorless oil by the same procedure described for **2h** using 4-hydroxybenzaldehyde (3.0 g, 25 mmol) and 2-[(propionyloxy)methyl-hydrogen]-1,3-propanediol diacetate (6.5 g, 25 mmol) as reaction agents. ESI-MS: *m/z* 225 (M − 1). IR (KBr, cm^{-1}) *ν*: 3425, 2935, 1687, 1605, 1509, 1229,

1162, 1068, 825; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 9.75 (s, 1H, CHO), 7.73 (d, 2H, J = 8.8 Hz, ArH), 7.08 (d, 2H, J = 8.8 Hz, ArH), 5.24 (s, 2H, CH_2), 3.82 (m, 1H, CH), 3.65 (m, 2H, CH_2), 3.53 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 190.6, 162.2, 131.9, 130.5, 116.1, 92.7, 80.7, 62.3; Anal. Calcd for $C_{11}H_{14}O_5$: C, 58.41; H, 6.19; found: C, 58.58; H, 6.31.

*5.2.10. 4-(2-Hydroxyethoxy)benzaldehyde (**3a**)*

A mixture of 4-hydroxybenzaldehyde (1.22 g, 10 mmol), 2-chloroethanol (3 ml, 15 mmol), 1,4-dioxane (15 ml), sodium hydroxide (4.4 g, 110 mmol) and water (5 ml) was refluxed for 3 h. The solvent was removed under reduced pressure and the residue was extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 1:3) to afford compound **3a** (1.35 g, 81%) as a yellow oil. ESI-MS: m/z 167(M + 1). IR (KBr, cm^{-1}) ν: 3370, 1684, 1600, 1509, 1309, 1164, 1112, 1034, 839; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 9.89 (s, 1H, CHO), 7.79(d, J = 8.4 Hz, 2H, ArH), 7.32 (d, J = 8.4 Hz, 2H, ArH), 4.75 (t, J = 4.8 Hz, 2H, CH_2), 4.42 (t, J = 4.8 Hz, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 190.2, 163.3, 131.5, 130.1, 114.4, 70.1, 61.1; Anal. Calcd for $C_9H_{10}O_3$: C, 65.06; H, 6.02; found: C, 65.18; H, 6.10.

*5.2.11. 2-(4-Formylphenoxy)ethyl dihydrogen phosphate (**3b**)*

A mixture of compound **3a** (1.66 g, 10 mmol), triethylamine (3.03 g, 30 mmol) and dichloroethane (40 ml) was cooled to 0 °C and treated dropwise with a solution of phosphorus oxychloride (3.04 g, 20 mmol) in dichloroethane and then stirred for 1.5 h. After the solvent evaporating, 1 M NaOH solution (20 mL) was added dropwise and stirred at room temperature for 5 h. The resulting mixture was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 1:5) to afford compound **3b** (1.25 g, 51%) as yellow oil. ESI-MS: m/z 247 (M + 1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 9.88 (s, 1H, CHO), 7.85 (d, J = 8.9 Hz, 2H, ArH), 7.02 (d, J = 8.9 Hz, 2H, ArH), 4.32 (t, J = 5.3 Hz, 2H, CH_2), 3.86 (t, J = 5.3 Hz, 2H, CH_2), 1.26 (s, OH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 190.5, 163.1, 131.9, 130.4, 114.8, 69.9, 65.4; Anal. Calcd for $C_9H_{11}O_6$ P: C, 43.91; H, 4.50; found: C, 43.88; H, 4.32.

*5.2.12. 2-(4-Formylphenoxy)ethyl dimethyl phosphate (**3c**)*

According to the same procedure described for **3b**, compound **3c** (1.10 g, 40%) was obtained as yellow oil by the esterification with anhydrous methanol (50 ml) and sodium methoxide (20 ml, 0.2 M). ESI-MS: m/z 297 (M + Na). IR (KBr, cm^{-1}) ν: 3475, 2958, 2856, 2742, 1688, 1600, 1510, 1457, 1262, 1168, 1039, 983, 847; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.88 (s, 1H, CHO), 7.85(d, J = 8.5 Hz, 2H, ArH), 7.03 (d, J = 8.5 Hz, 2H, ArH), 4.42 (m, 2H, CH_2), 4.28 (m, 2H, CH_2), 3.81 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 190.3, 163.2, 132.4, 130.6, 114.2, 70.1, 65.5, 55.6, 55.3; ^{31}P NMR [a solution (H_3PO_4:H_2O = 1:10) as an external standard] δ: 2.32 ppm. Anal. Calcd for $C_{11}H_{15}O_6P$: C, 48.18; H, 5.47; found: C, 48.01; H, 5.65.

*5.2.13. 2-(4-Formylphenoxy)ethyl diethoxyl phosphate (**3d**)*

According to the same procedure described for **3b**, compound **3d** (1.27 g, 42%) was obtained as yellow oil by the esterification with anhydrous ethanol (50 ml) and sodium ethoxide (20 ml, 0.2 M). ESI-MS: m/z 303 (M + 1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 9.86 (s, 1H, CHO), 7.83 (d, J = 8.9 Hz, 2H, ArH), 7.01 (d, J = 8.9 Hz, 2H, ArH), 4.30 (t, J = 5.4 Hz, 2H, CH_2), 4.11 (m, 4H, CH_2), 3.85 (t, J = 5.4 Hz, 2H, CH_2); 1.27 (m, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 190.6, 163.3, 131.6, 130.0, 114.6, 70.3, 64.8, 63.5, 63.2, 15.1, 14.9; Anal. Calcd for $C_{13}H_{19}O_6P$: C, 51.66; H, 6.34; found: C, 51.82; H, 6.28.

*5.2.14. 3-[(4-Formylphenoxy)ethoxycarbonyl]propanoic acid (**3e**)*

A mixture of triethylamine (2.0 ml), anhydrous dichloromethane (20 ml) and compound **3a** (1.66 g, 10 mmol) was added dropwise into succinic anhydride (1.1 g, 11 mmol) in anhydrous dichloromethane (50 ml). The solution was stirred at 100 °C for 0.5 h and then treated with 4-dimethylaminopyridine and refluxed for 4 h. The solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography (ethyl acetate:petroleum ether = 1:6) to yield yellow solid (1.3 g, 50%). mp 97–99 °C. ESI-MS: m/z 265 (M − 1). IR (KBr, cm^{-1}) ν: 3452, 2963, 1731, 1666, 1595, 1509, 1407, 1347, 1315, 1263, 1211, 1162, 834; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 9.88 (s, 1H, CHO), 7.85 (d, J = 8.7 Hz, 2H, ArH), 7.03 (d, J = 8.7 Hz, 2H, ArH), 4.50 (t, J = 4.5 Hz, 2H, CH_2); 4.26 (t, J = 4.5 Hz, 2H, CH_2), 2.70 (s, 4H, 2 × CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 191.1, 177.7, 172.2, 163.6, 132.3, 130.4, 115.3, 115.1, 66.4, 63.0, 29.2, 29.1; Anal. Calcd for $C_{13}H_{14}O_6$: C, 58.65; H 5.26; found: C, 58.32; H, 5.55.

5.3. Tyrosinase assay

The spectrophotometric assay for tyrosinase was performed according to the method reported by our groups [22,23] with some slight modifications. Briefly, all the synthesized compounds were screened for the diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All the compounds were dissolved in DMSO. The final concentration of DMSO in the test solution was 2.0%. Phosphate buffer, pH 6.8, was used to dilute the DMSO stock solution of test compounds. Thirty units of mushroom tyrosinase (0.5 mg/ml) were first pre-incubated with the compounds, in 50 mM phosphate buffer (pH 6.8), for 10 min at 25 °C. Then the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm of formation of the DOPAchrome for 1 min. The measurement was performed in triplicate for each concentration and averaged before further calculation. IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose–response curves. 4-Methoxycinnamic acid and 4-hydroxybenzaldehyde were used as standard inhibitors for the tyrosinase.

5.4. Inhibition kinetics of compound **3c**

The determination of inhibitor kinetics was performed by modification of the above-mentioned method: for each of three different inhibitor concentrations (0, 12.94 μM and 25.88 μM, respectively.) L-DOPA concentration was varied (0, 5, 10, 15, 20 and 25 μL). Pre-incubation and measurement time was the same as above. Maximal initial velocity $v = \delta A/\delta t$ was determined from initial linear portion of absorbance between 0 and 60 s after addition of mushroom tyrosinase. The inhibition type on the enzyme was assayed by Lineweaver–Burk plots, and the inhibition constant was determined by the second plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of compound.

5.5. Preliminary acute toxicity assays in mice

Acute toxicity assay was performed according to the method described by Shim et al. [24] with some slight modifications. Briefly, ten mice (specific pathogen free, 4 weeks of age) were acclimated for a week in the housing: Temperature was maintained at 20 ± 2 °C and relative humidity was 50 ± 10%. Body weight of the mice at study beginning was 20 ± 2 g. Mice were fasted for 4 h prior to oral administration. Compound **3c** was dissolved in CMC (carboxymethyl cellulose). Constant volume (10 ml/kg body weight) containing the concentration of compound **3c** (160 mg/ml) was orally administered to the mice to achieve the dose of 1600 mg/kg. CMC was used as negative control. Feeds and water were provided after 4 h of oral administration. Clinical symptom was observed

once a day for 20 days. All animals were sacrificed at the end of the experimental period and checked macroscopically for possible damage to organs, such as heart, liver and kidneys.

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