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Synthesis and Antityrosinase Activities of Alkyl 3,4-Dihydroxybenzoates

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ABSTRACT: In insects, tyrosinase plays important roles in normal developmental processes, such as cuticular tanning, scleration, wound healing, production of opsonins, encapsulation and nodule formation for defense against foreign pathogens. Thus, tyrosinase may be regarded as a potential candidate for novel bioinsecticide development. A family of alkyl 3,4-dihydroxybenzoates (C_6-C_9), new tyrosinsase inhibitors, were synthesized. Their inhibitory effects on the activity of tyrosinase have been investigated. The results showed all of them could inhibit the activity of tyrosianse effectively. The order of potency was nonyl 3,4-dihydroxybenzoate (C_9DB) > octyl 3,4-dihydroxybenzoate(C_8DB) > heptyl 3,4-dihydroxybenzoate(C_7DB) > hexyl 3,4-dihydroxybenzoate (C_6DB). The kinetic analysis of these four compounds on tyrosinase was taken to expound their inhibitory mechanism. The research of the control of insects in agriculture was taken as C_6DB for example. C_6DB could inhibit the development and molting of *Plutella xylostella* effectively. To clarify its insecticidal mechanism, we researched the expression of tyrosinase in the *P. xylostella* treated with C_6DB by real-time quantitative PCR. The results showed C_6DB could inhibit the expression of tyrosinase in the *P. xylostella* as expected.

KEYWORDS: synthesize, tyrosinase, inhibition kinetics, *Plutella xylostella*, real-time quantitative PCR

INTRODUCTION

Tyrosinase (EC 1.14.18.1), a copper containing enzyme, is ubiquitously distributed in microorganisms, animals, insects and plants.[1,2] It is essential for the formation of melanin and various other functions. It can catalyze both the hydroxylation of monophenol to *o*-diphenol (monophenolase activity) and the oxidation of diphenol to *o*-quinones (diphenolase activity) by using molecular oxygen followed by a series of nonenzymatic steps resulting in the formation of melanin.[3−5] In the bacterium, melanin can protect the bacterial cells and spores against UV radiation.[6−8] Meanwhile melanin can bind heavy metals that are toxic to the cells;[8] it is important to bacterial protection mechanisms. In insects, tyrosinase plays important roles in normal developmental processes, such as cuticular tanning, scleration, wound healing, production of opsonins, encapsulation and nodule formation for defense against foreign pathogens.[9] The inhibition of activity of tyrosinase could lead to abrogation of insect defense mechanisms or abnormal body softening, both of which could lead to pest control. Tyrosinase may be regarded as a potential candidate for novel bioinsecticide development.

In our previous research, a great many tyrosinase inhibitors were screened from natural materials and synthetic methods. We have reported some tyrosinase inhibitors, such as *p*-alkylbenzoic acids;[10] hinokitiol;[11] fatty acids;[12] 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid;[13] α-cyano-4-hydroxycinnamic acid;[14] cefazolin and cefodizime;[15] methyl *trans*-cinnamate;[16] *trans*-cinnamaldehyde thiosemicarbazone;[17] all of these compounds showed inhibitory effects on the activity of tyrosinase. In this present work, we synthesized a family of alkyl 3,4-dihydroxybenzoates (C_6-C_9), new tyrosinsase inhibitors, after the research of the structure activity relationship of tyrosinase inhibitors and the safety considerations. We studied the inhibition of alkyl 3,4-dihydroxybenzoates on mushroom tyrosinase and their effects on the expression of tyrosinase in *Plutella xylostella*. It may provide the basis for developing novel insecticides.

MATERIALS AND METHODS

Insects. Specimens of *P. xylostella* were obtained from Bio-Pesticide Engineering Research Center of Hubei Province. Larvas were raised with the feed from the Bio-Pesticide Engineering Research Center of Hubei Province at 27 ± 2 °C, 70% humidity, over a 14:10 h light:dark photoperiod.

Reagents. Tyrosinase (EC 1.14.18.1) from mushroom was the product of Sigma Chemical Co (St. Louis, MO, USA). The specific activity of the enzyme is 6680 U/mg. RNAiso Plus; PrimeScript RT reagent Kit and SYBR Premix Ex Taq (Perfect Real Time) were purchased from TaKaRa Co. L-3,4-Dihydroxyphenylalanine (L-DOPA), 4-dimethylaminopyridine (DMAP) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). The feed of *P. xylostella* was purchased from Bio-Pesticide Engineering Research Center of Hubei Province. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

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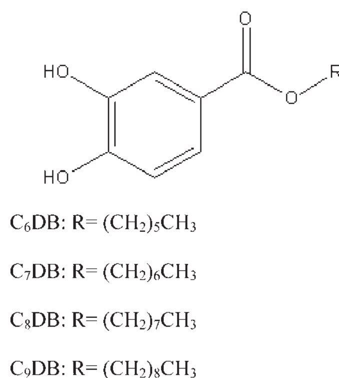

Figure 1. Chemical structures of alkyl 3,4-dihydroxybenzoates.

hydrochloride (EDC·HCl) and other reagents were local and of analytical grade. The water used was redistilled and ion-free.

Synthesis. A family of alkyl 3,4-dihydroxybenzoates (C_6-C_9) (the chemical structures are shown in Figure 1) were synthesized by esterification reaction with EDC·HCl and DMAP as catalysts. In brief, a solution of 3,4-dihydroxybenzoic acid (1.0 mmol) and corresponding alcohol (1.2 mmol) was added to a solution of EDC·HCl (1.2 mmol) and DMAP. After being stirred overnight at room temperature, the solvent was removed under reduced pressure. The residue was extracted with citric acid and ethyl acetate several times and filtered. The organic layer was dried over Na_2SO_4 and evaporated. The crude products were purified by silica gel chromatography. The structures of these esters were established by spectroscopic methods (MS and NMR).

Enzyme Assay. The enzyme activity assay was performed as reported by Chen et al.[18] In this investigation, L-DOPA was used as a substrate for the activity assay of tyrosinase. The reaction media (3 mL) for activity assay contained 0.5 mM L-DOPA in 50 mM sodium phosphate buffer (pH 6.8). The final concentration of mushroom tyrosinase was 6.67 μg/mL for the *o*-diphenolase activity. The alkyl 3,4-dihydroxybenzoates were dissolved in DMSO and diluted to some appropriate concentrations. The final concentration of DMSO in the test solution was 3.3%. The controls, without inhibitors but containing 3.3% DMSO in the reaction media, were routinely carried out. The reaction was carried out at a constant temperature of 30 °C. The inhibitory effect of the alkyl 3,4-dihydroxybenzoates was expressed as the concentration that inhibited 50% of the enzyme activity (IC_{50}). The inhibition type was assayed by the Lineweaver−Burk plot, and the inhibition constant was determined by the secondary plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor. A Beckman UV-650 spectrophotometer was used for absorbance and kinetic parameters.

The Expression of Tyrosinase in *P. xylostella* Treated with C_6DB by Real-Time Quantitative PCR. The C_6DB was dissolved in acetone and then diluted to five different concentrations (40, 20, 5, and 2.5 mg/mL). The feed was transferred to the 24-well plate, and then five different concentrations of C_6DB were spread well on the surface of the feed, respectively. The newly molted second-instar *P. xylostella* were gently removed to the feed after the acetone was completely evaporated. Control was treated with the same volume of the acetone. *P. xylostella* were placed in an air-conditioned room at 27.0 ± 2.0 °C and monitored daily for 3 days. Measurement was performed after 72 h of treatment. Real-time quantitative PCR was performed to determine expression of *P. xylostella* prophenoloxidase (PxPPO) after the treatment with C_6DB. Total RNA was extracted from *P. xylostella* using the RNAiso Plus (TaKaRa Code: D9108A) following the manufacturer's instructions and then reverse transcribed to cDNA using the PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa Code: DRR037S). The conditions for reverse transcription include 37 °C for 15 min (reverse transcription reaction) and one cycle at 85 °C for 5 s (denaturation of reverse transcriptase).[19] The primers used for PxPPO were 5′-AGCA-GATGGCTGACGAGG-3′ and 5′-CCGCAAAGTTGGGAATGG-3′ for the forward and reverse respectively. GAPDH was used as the reference to normalize the expression levels between the samples. The primers used for real-time quantitative PCR were 5′-CAGTGCC-GATGCACCTATGTTC-3′ (forward) and 5′- AAGTTGTCGTT-GAGGGAGATGCC-3′ (reverse).[20] Real-time quantitative PCR was carried out using the SYBR Premix Ex Taq (Perfect Real Time) (TaKaRa Code: DRR041A). PCR was performed in a Rotor-Gene 3000 (Corbett Research, Australia) according to the following protocol: 10 s at 95 °C, 1 cycle; 5 s at 95 °C and 20 s at 60 °C, 45 cycles. Fluorescence was detected at the annealing stage of each cycle. A melting curve was generated during the reactions to check for the possibility of primer−dimer formation. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA level of each gene.[21]

RESULTS AND DISCUSSION

Synthesis of Alkyl 3,4-Dihydroxybenzoates. Hexyl 3,4-dihydroxybenzoate (C_6DB) obtained was a white powder. ^{1}H NMR (600 MHz, dimethyl sulfoxide-d_6): δ 9.55 (br s, 2H), 7.36 (d, J = 2.0 Hz, 1H), 7.32 (dd, J = 2.0, 8.3 Hz, 1H), 6.81 (d, J = 8.3 Hz, 1H), 4.17 (t, J = 6.5 Hz, 2H), 1,66 (m, 2H), 1.35 (m, 2H), 1.27 (m, 4H), 0.85 (m, 3H). ^{13}C NMR (600 MHz, dimethyl sulfoxide-d_6): δ 166.17, 150.80, 145.50, 122.16, 121.28, 116.70, 115.72, 64.44, 31.35, 25.70, 25.64, 22.47, 14.29. ESI-MS: m/z 238.1 (M + Na$^+$).

Heptyl 3,4-dihydroxybenzoate(C_7DB) was obtained as a white powder. ^{1}H NMR (600 MHz, dimethyl sulfoxide-d_6): δ 9.70 (br s, 1H), 9.41 (br s, 1H), 7.35 (d, J = 1.9 Hz, 1H), 7.31 (dd, J = 2.0, 8.2 Hz, 1H), 6.81 (d, J = 8.3 Hz, 1H), 4.17 (t, J = 6.5 Hz, 2H), 1,67 (m, 2H), 1.37 (m, 8H), 0.85 (m, 3H). ^{13}C NMR (600 MHz, dimethyl sulfoxide-d_6): δ 166.17, 150.81, 145.50, 122.16, 121.28, 116.71, 115.74, 64.45, 31.64, 28.80, 28.75, 25.95, 22.48, 14.35. ESI-MS: m/z 252.1 (M + Na$^+$).

Octyl 3,4-dihydroxybenzoate(C_8DB) was obtained as a white powder. ^{1}H NMR (600 MHz, dimethyl sulfoxide-d_6): δ 9.76 (s, 1H), 9.35 (s, 1H), 7.34 (s, 1H), 7.30 (d, J = 8.3 Hz, 1H), 6.80 (d, J = 8.2 Hz, 1H), 4.18 (t, J = 6.4 Hz, 2H), 1,66 (m, 2H), 1.36 (m, 10H), 0.85 (m, 3H). ^{13}C NMR (600 MHz, dimethyl sulfoxide-d_6): δ 166.17, 150.81, 145.50, 122.17, 121.27, 116.70, 115.76, 64.46, 31.66, 29.08, 29.05, 28.72, 25.98, 22.51, 14.39. ESI-MS: m/z 266.2 (M + Na$^+$).

Nonyl 3,4-dihydroxybenzoate (C_9DB) was obtained as a white powder. ^{1}H NMR (600 MHz, dimethyl dulfoxide-d_6): δ 9.77 (s, 1H), 9.35 (s, 1H), 7.35 (d, J = 1.9 Hz, 1H), 7.30 (dd, J = 1.9, 8.2 Hz, 1H), 6.80 (d, J = 8.3 Hz, 1H), 4.17 (t, J = 6.5 Hz, 2H), 1,67 (m, 2H), 1.38 (m, 12H), 0.86 (m, 3H). ^{13}C NMR (600 MHz, dimethyl sulfoxide-d_6): δ 166.17, 150.81, 145.51, 122.16, 121.27, 116.70, 115.75, 64.45, 31.70, 29.35, 29.12, 29.08, 28.72, 25.97, 22.54, 14.39. ESI-MS: m/z 280.1 (M + Na$^+$).

Concentration Effects of Alkyl 3,4-Dihydroxybenzoates on the Activity of Mushroom Tyrosinase. The activity of the mushroom tyrosinase was assayed using L-DOPA as substrate. Alkyl 3,4-dihydroxybenzoates were tested for the inhibitory effect on the oxidation of L-DOPA catalyzed by mushroom tyrosinase. The enzyme activity was monitored by following the increasing absorbance at 475 nm accompanying the oxidation of L-DOPA. The progress curve of enzyme reaction was a line passing through the origin without lag period. The value of the slope of the line indicated the activity of tyrosinase. The activity

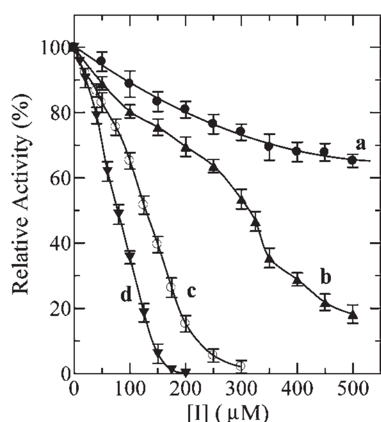

Figure 2. Effects of alkyl 3,4-dihydroxybenzoates on the activity of mushroom tyrosinase for the oxidation of L-DOPA. Curves a, b, c and d denote the compounds C_6DB, C_7DB, C_8DB and C_9DB.

Table 1. Inhibition Constants of Alkyl 3,4-Dihydroxybenzoates on Mushroom Tyrosinase

	IC_{50} (μM)				
compd	tested	theor	mechanism[a]	type	K_{IS} (μM)
C_6DB		909	R	UC	389
C_7DB	316	315	R	UC	135
C_8DB	129		R/IR[b]	UC	
C_9DB	81		IR	not tested	

[a] R: reversible inhibition. IR: irreversible inhibition. [b] Reversible at $[I] \leq 50$ μM, irreversible at $[I] \geq 75$ μM.

of mushroom tyrosinase decreased with increasing concentrations of alkyl 3,4-dihydroxybenzoates as shown in Figure 2. The concentrations leading to 50% activity loss (IC_{50}) obtained from Figure 2 are summarized in Table 1 for comparison. The inhibitory effects of C_6DB, C_7DB, C_8DB and C_9DB were in the order $C_6DB < C_7DB < C_8DB < C_9DB$. The inhibitory effects of these four compounds were potentiated with increasing length of hydrocarbon chain.

Inhibitory Mechanism of Alkyl 3,4-Dihydroxybenzoates on Mushroom Tyrosinase. Figure 3 shows the inhibition mechanism of alkyl 3,4-dihydroxybenzoates on mushroom tyrosinase for the oxidation of L-DOPA. The plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of compounds gave a family of straight lines. If all straight lines passed through the origin, the inhibition was reversible, but if they did not pass through the origin and were parallel lines, the inhibition was irreversible. From Figure 3, we found that C_6DB and C_7DB were reversible inhibitors and C_9DB was an irreversible inhibitor. When the concentration of C_8DB was lower than 50 μM, it showed a reversible inhibitory mechanism, but when the concentration was above 75 μM, the inhibitory mechanism of C_8DB was irreversible. The kinetic constants obtained are listed in Table 1.

Inhibition Type and Inhibition Constants of Alkyl 3,4-Dihydroxybenzoates on Mushroom Tyrosinase. The kinetic behaviors of the oxidation of L-DOPA, catalyzed by mushroom tyrosinase at different concentrations of alky 3,4-dihydroxybenzoates, were studied. Figure 4 shows the double-reciprocal plots of the enzyme inhibited by C_7DB. The results showed that

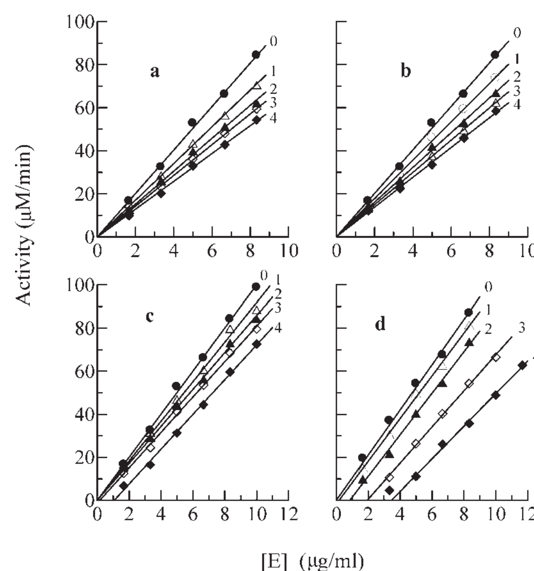

Figure 3. Determination of the inhibitory mechanism of alkyl 3,4-dihydroxybenzoates on mushroom tyrosinase. Panels a, b, c and d denote compounds C_6DB, C_7DB, C_8DB and C_9DB, respectively. The concentrations of C_6DB and for C_7DB of lines 0−4 were 0, 50, 100, 150, and 200 μM, respectively. The concentrations of C_8DB for curves 0−4 were 0, 25, 50, 75, and 100 μM, respectively. The concentrations of C_9DB for curves 0−4 were 0, 20, 40, 60, and 80 μM, respectively.

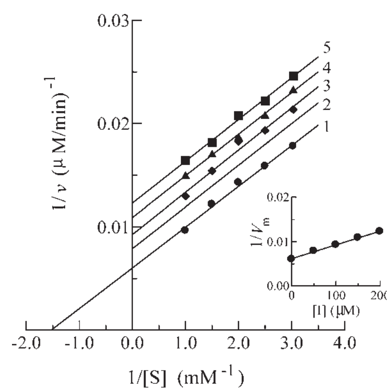

Figure 4. Lineweaver−Burk plots for inhibition of C_7DB on mushroom tyrosinase for the catalysis of L-DOPA at 30 °C, pH 6.8. The concentrations of C_7DB for lines 1−5 were 0, 50, 100, 150, and 200 μM, respectively. The inset represents the secondary plot of K_m versus concentration of C_7DB to determine the inhibition constant K_{IS}.

the plots of $1/v$ versus $1/[S]$ gave a family of parallel straight lines with the same slopes. Accompanying the increase of the inhibitor concentration, the values of both K_m and V_m increased, but the ratio of K_m/V_m remained unchanged. The slopes were independent of the concentration of C_7DB, which indicated that C_7DB was an uncompetitive inhibitor on the enzyme. When the concentration was lower than 50 μM, the inhibition type of C_6DB and C_8DB was the same, belonging to be uncompetitive. The results indicated that 3,4-dihydroxybenzoates could only bind with the enzyme−substrate complex, not with the free enzyme. The inhibition constant, K_{IS}, was obtained from a plot of the vertical intercept ($1/V_m$) versus the concentration of C_7DB, which is linear, as shown in the inset in Figure 4. The inhibition constants are summarized in Table 1 for comparison.

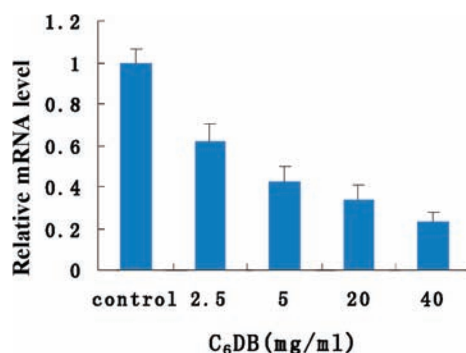

Figure 5. Real-time quantitative PCR analysis of PxPPO2 transcripts with C_6DB treated. PxPP02 transcripts were determined from the whole body at 72 h after treament with C_6DB at concentrations of 2.5, 5, 20, and 40 mg/mL. All the data were normalized relative to GAPDH.

The Expression of Tyrosinase in *P. xylostella* Treated with C_6DB by Real-Time Quantitative PCR. C_6DB showed an inhibitory effect against mushroom tyrosinase among these four compounds with IC_{50} of 0.91 mM; it was a reversible and uncompetitive inhibitor. The inhibition constant (K_{IS}) was determined to be 0.39 mM. We explored its insecticidal action from the effect on the expression of tyrosinase in *P. xylostella*. From the results, we found that it could effectively inhibit the development and molting of *P. xylostella*. With the increasing of the concentration of C_6DB, the inhibitory effects become stronger. The *P. xylostella* insects treated with C_6DB in the concentration of 40 mg/mL were 0.3 cm long on average after 3 days; with the control they were 0.95 cm on average. We analyzed the expression of tyrosinase in *P. xylostella* treated with C_6DB by real-time quantitative PCR furthermore. The insect tyrosinase was labile and easy to inactivate during purification. Its inactive precursor, prophenoloxidase (PPO), was more stable. Tyrosinase could be activated from PPO by specific proteolysis via a serine protease cascade[22] in *P. xylostella*, and the cDNA encoding PPO from *P. xylostella* has been cloned. In the present work, we investigate the influence of C_6DB on the relative amount of PxPPO transcripts by real-time quantitative PCR. As Figure 5 shows, PxPPO transcript levels were gradually decreased with increasing concentration of C_6DB as compared with control samples. It indicated that C_6DB could inhibit the expression of tyrosinase in *P. xylostella* to inhibit the molting of *P. xylostella*. It was a potent biological insecticide.

Tyrosinase was a key enzyme in the browning in the fruits and vegetables and the normal developmental processes of insects; all of C_6DB, C_7DB, and C_9DB could inhibit the activity of tyrosinase effectively. As the insecticidal activity of C_6DB shows, it could inhibit the expression of tyrosinase in *P. xylostella* to inhibit the molting of *P. xylostella*. All of these data provide a basis to develop a novel, effective and safe biological insecticide. They also contribute to the development of an antibrowning food additive.

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Author Contributions

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ABBREVIATIONS USED

PPO, prophenoloxidase; PxPPO, *Plutella xylostella* prophenoloxidase; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; DMAP, 4-dimethylaminopyridine; L-DOPA, L-3,4-dihydroxyphenylalanine; DMSO, dimethyl sulfoxide; IC_{50}, the inhibitor concentration leading to 50% activity loss; K_{IS}, equilibrium constant of the inhibitor combining with the enzyme−substrate complex

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