Regular Article

Inhibition of Mushroom Tyrosinase Activity by Orsellinates

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Several applications have been proposed for tyrosinase inhibitors in the pharmaceutical, food bioprocessing, and environmental industries. However, only a few compounds are known to serve as effective tyrosinase inhibitors. This study evaluated the tyrosinase-related activity of resorcinol (1), orcinol (2) lecanoric acid (3), and derivatives of this acid (4–15). Subjected to alcoholysis, lecanoric acid (3), a depside isolated from the lichen *Parmotrema tinctorum*, produces orsellinic acid (2,4-dihydroxy-6-methylbenzoic acid) (4) and orsellinates (2,4-dihydroxy-6-methyl benzoates) (5–15). At 0.50 mM, methyl (5), ethyl (6), *n*-propyl (7), *tert*-butyl (11), and *n*-cetyl orsellinates (15) acted as tyrosinase activators, whereas *n*-butyl (8), *iso*-propyl (9), *sec*-butyl (10), *n*-pentyl (12), *n*-hexyl (13), and *n*-octyl orsellinates (14) behaved as inhibitors. Tyrosinase inhibition rose with chain elongation-*n*-butyl (8)<*n*-pentyl (12)<*n*-hexyl (13)<*n*-octyl orsellinates (14)-suggesting that the enzyme site can accept an eight-carbon alkyl chain. A kinetic study of *n*-octyl orsellinate (14) revealed uncompetitive inhibition of tyrosinase, with an inhibition constant of 0.99 mM.

Key words tyrosinase; orsellinate; lecanoric acid; *Parmotrema tinctorum*

Tyrosinase (EC 1.14.18.1) is a copper enzyme that catalyzes both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones.[1,2] *o*-Quinones are highly reactive compounds that can polymerize spontaneously to form high-molecular-weight compounds, such as melanin. In humans, melanin protects the skin against UV radiation, but its overproduction can result in malignant melanoma.[3] In foods, *o*-quinones can react with amino acids and/or proteins, conferring enhanced brownish coloration and affecting the organoleptic properties and appearance of foodstuffs.[4]

Several applications have been proposed for tyrosinase inhibitors in the pharmaceutical, food bioprocessing, and environmental industries[5]—*e.g.*, for medicinal purposes, such as in the treatment of neurodegenerative diseases,[6] or to improve the quality and shelf life of food products by inhibiting enzymatic browning.[7] Tyrosinase inhibitors are often structurally analogous to phenolic substrates, which generally show competitive inhibition. A number of naturally occurring tyrosinase inhibitors have been described, mostly phenols and polyphenols,[8] resorcinol derivatives,[9–11] benzoic acid and pyridine derivatives,[12,13] gallate derivatives,[14] and vanillin derivatives.[15] However, only a few compounds are known to serve as effective tyrosinase inhibitors, owing to high toxicity, low activity, or economic factors. Potentially active compounds, such as kojic acid and arbutin, have not shown the desired clinical effects.[16]

Recently, lichen extracts have been found to inhibit tyrosinase. *Cladia aggregata*, *Cladonia dimorphoclada*, *Stereocaulon ramulosum*, and *Stereocaulon microcarpum* extracts have proven active against mushroom tyrosinase.[17] In the present investigation, the activity of resorcinol (**1**), orcinol (**2**), lecanoric acid (**3**), and derivatives of this acid (**4**–**15**) against mushroom tyrosinase was evaluated. Subjected to alcoholysis, lecanoric acid, a lichen depside isolated from *Parmotrema tinctorum*, yields orsellinic acid (2,4-dihydroxy-6-methylbenzoic acid) (**4**) and orsellinates (2,4-dihydroxy-6-methyl benzoates) (**5**–**15**). Orsellinates have been evaluated against microorganisms[18] and *Artemia salina*,[19] and also investigated for their antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH)[20] and for antitumor properties.[21]

The purpose of the present study was to investigate the effect that resorcinol (**1**), orcinol (**2**), lecanoric acid (**3**), orsellinic acid (**4**), and methyl (**5**), ethyl (**6**), *n*-propyl (**7**), *n*-butyl (**8**), *iso*-propyl (**9**), *sec*-butyl (**10**), *tert*-butyl (**11**), *n*-pentyl (**12**), *n*-hexyl (**13**), *n*-octyl (**14**), and *n*-cetyl orsellinates (**15**) exert on tyrosinase activity. The inhibition mechanism involved in *n*-octyl orsellinate (**14**) was also investigated. To our knowledge, this is the first report on the anti-tyrosinase behavior of these compounds.

Results and Discussion

Tyrosinase Activation or Inhibition All compounds (Fig. 1) were evaluated at 0.50 mM and the results are expressed as percent activation (*A*) or inhibition (*I*) (Table 1). The inhibitory effect decreases with rising numerical values. Resorcinol inhibited tyrosinase activity by 47.96%. Introducing a methyl group into position 5 of resorcinol to yield orcinol led to a slight decrease in inhibition (50.79%). Khatib *et al.*[10] reported that tyrosinase inhibitors containing a resorcinol subunit are typically highly active, and 2,4-resorcinol derivatives are significantly more potent than 3,5-substituted counterparts. Introduction of a carboxyl into position 4 of orcinol to produce orsellinic acid slightly diminished diphenolase inhibition to 63.35% an inhibitory level still higher than that of 82.69% observed for lecanoric acid.

Modifying the alkyl chain of orsellinic acid had a pronounced effect on the behavior of derivative **5**–**15** toward mushroom tyrosinase. Orsellinates with small alkyl chains, such as methyl (110.10%), ethyl (114.49%), and *n*-propyl (125.76%) orsellinates, activated the enzyme, enhancing its diphenolase activity. In summary, orsellinates with small alkyl chains function as activators, and elongation of the alkyl chain causes an inhibitory activity to manifest, in contrast with alkylgallates, which act as inhibitors regardless of alkyl chain

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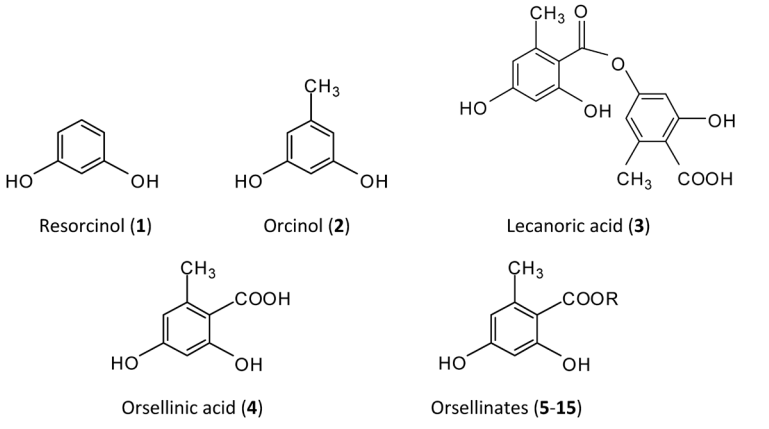

Fig. 1. Structures of the Compounds Investigated

Table 1. Effect of Compounds **1**–**15** on Tyrosinase

Compound	Tyrosinase activity (%)
Resorcinol (**1**)[a]	47.96
Orcinol (**2**)[a]	50.79
Lecanoric acid (**3**)[b]	82.69
Orsellinic acid (**4**)[a]	63.35
Orsellinates	
Methyl (**5**)[a]	110.10
Ethyl (**6**)[a]	114.49
n-Propyl (**7**)[a]	125.76
n-Butyl (**8**)[a]	90.59
iso-Propyl (**9**)[a]	88.06
sec-Butyl (**10**)[a]	57.30
tert-Butyl (**11**)[a]	111.05
n-Pentyl (**12**)[a]	65.92
n-Hexyl (**13**)[a]	58.55
n-Octyl (**14**)[b]	45.62
n-Cetyl (**15**)[b]	110.04

a) 5% and *b*) 15% of DMSO in reaction mixtures. Tyrosinase activity compared with a control reaction. For inhibitors, tyrosinase activity<100%; for activators, tyrosinase activity>100%.[24]

size.[4] The contrasting effects of alkyl chains in orsellinates and in gallates can be related to differences in the position of hydroxyl groups in orsellinates (2,4-dihydroxy-6-methyl benzoates) and gallates (3,4,5-trihydroxybenzoate) and to the presence of the methyl group in the latter. Previous reports have demonstrated a shift from activator to inhibitor, associated with substituent modification.[22] You *et al.* demonstrated that the introduction of a thiosemicarbazide group in aminoacetophenones transforms them from activators into inhibitors.[23]

Among linear-chain orsellinates, inhibitory activity rose with increasing number of carbons in the alkyl chain-namely, *n*-butyl (90.59%)<*n*-pentyl (65.92%)<*n*-hexyl (58.55%)<*n*-octyl orsellinate (45.62%)-corroborating the findings of Huang *et al.*,[13] who observed that the inhibitory properties of *p*-alkyl benzoic acids were potentiated by increasing the length of hydrocarbon chains. A similar result was obtained by Jiménez *et al.*,[9] who found 4-hexylresorcinol to be more active than 4-ethylresorcinol in preventing L-3,4-dihydroxyphenylalanine (L-DOPA) oxidation to DOPA-quinone. However, the enhancement of tyrosinase activity with increasing length of hydrocarbon chains is expected to peak at a linear alkyl chain of eight to ten carbons. Tyrosinase inhibition is likely related, at least in part, to hydrophobic interactions with the enzyme's hydrophobic domain.[24] This apparently holds true for orsellinates. Huang *et al.*[13] suggested that the enzyme's hydrophobic site may well accept an alkyl chain of eight carbons, which explains the enhanced activity observed for *n*-octyl orsellinate (45.62%).

Khatib *et al.*[10] suggested that the increasing inhibitory potency exhibited by 3-(2,4-dihydroxyphenyl) propionic esters does not necessarily correlate with longer alkyl chains, but may be related to the increasing volume of ester groups. Similar results were obtained by Kubo *et al.*[4] for esters of gallic acid. This can explain the higher inhibition activity observed for *sec*-butyl orsellinate (57.30%), relative to *n*-butyl orsellinate (90.59%). Inhibiting activity was also observed for *iso*-propyl orsellinate (88.06%), while the *n*-propyl counterpart proved a tyrosinase activator (125.76%). Unexpected results, however, were obtained for *tert*-butyl orsellinate (111.05%).

The Mechanism of Diphenolase Inhibition by *n*-Octyl Orsellinate Figure 2 depicts the behavior of diphenolase activity in the presence of *n*-octyl orsellinate at various concentrations—a family of straight lines intercepting the origin. Increasing inhibitor concentrations resulted in descending slopes, indicating reversible inhibition of mushroom tyrosinase by this orsellinate.

Double-reciprocal plots were used to investigate the mechanisms of mushroom tyrosinase inhibition by the same compound (Fig. 3). Plotting $1/V$ *vs.* $1/[S]$ yielded a family of parallel straight lines with equal slopes, indicating uncompetitive inhibition.[25] In uncompetitive inhibition, the inhibitor binds at a different site from the substrate and combines with the enzyme–substrate complex (*ES*), but not with the free enzyme (*E*).

The equilibrium constant for binding with the enzyme–sub-

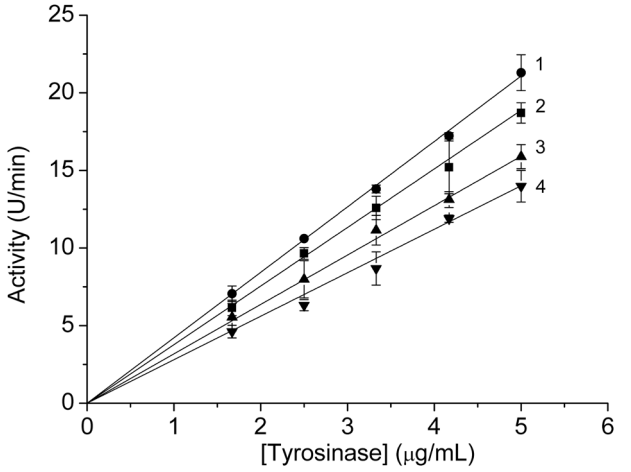

Fig. 2. Effect of *n*-Octyl Orsellinate (**14**) Concentration on Diphenolase Activity of Mushroom Tyrosinase

Curves 1–4 (for 0, 0.05, 0.10, and 0.15 mM *n*-octyl orsellinate, respectively) were obtained using linear regression.

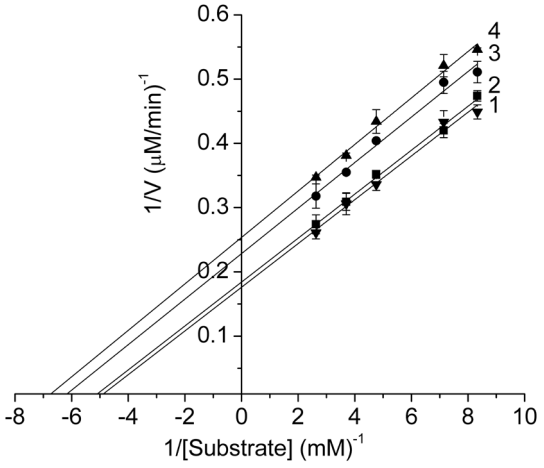

Fig. 3. Lineweaver–Burk Plots for Inhibition of Diphenolase Activity of Mushroom Tyrosinase by *n*-Octyl Orsellinate (**14**)

Curves 1–4 (0, 0.05, 0.17, and 0.28 mM *n*-octyl orsellinate, respectively) were obtained using linear regression.

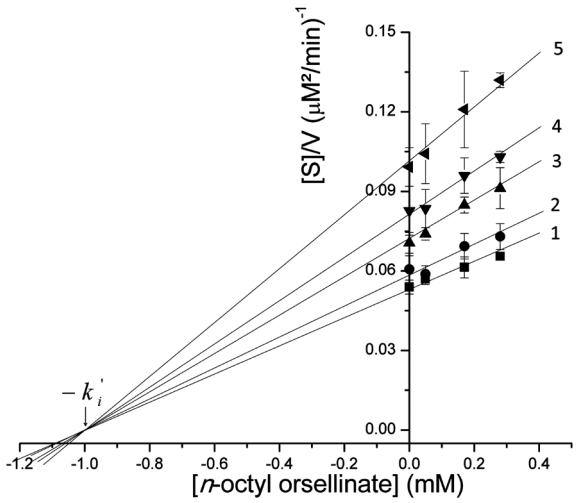

Fig. 4. Plot of [S]/*V vs.* Concentration of *n*-Octyl Orsellinate (**14**), to Determine the Enzyme–Substrate–Inhibitor Complex (KESI)

Curves 1–5 (0.12, 0.14, 0.21, 0.27 and 0.38 mM L-DOPA, respectively) were obtained using linear regression.

strate–inhibitor complex (KESI) was obtained by regression from 1/*V vs.* inhibitor concentration (Fig. 4). The inhibition constant obtained for *n*-octyl orsellinate was 0.99 mM.

Conclusion

The activity of orsellinates against mushroom tyrosinase was investigated. The behavior of orsellinates differed from those of orcinol and orsellinic acid. This difference was ascribed to the presence of an ester group *ortho*-positioned to a methyl group, as well as to the presence of a hydroxyl in the structure of orsellinates, as compared with orcinol, or to esterification of the carboxyl group of orsellinic acid. Tested at 0.50 mM, methyl, ethyl, *n*-propyl, *tert*-butyl, and *n*-cetyl orsellinates activated mushroom tyrosinase, while *n*-butyl, *iso*-propyl, *sec*-butyl, *n*-pentyl, *n*-hexyl, and *n*-octyl orsellinates acted as inhibitors. Inhibition increased with chain elongation (*n*-butyl<*n*-pentyl<*n*-hexyl<*n*-octyl orsellinates), suggesting that the hydrophobic site of the enzyme can accept an alkyl chain of eight carbons. The kinetic study of *n*-octyl orsellinate revealed uncompetitive inhibition of tyrosinase, with an inhibition constant of 0.99 mM.

Experimental

General Procedures NMR spectra were recorded using a Bruker DPX-300 spectrometer operating at 300.13 MHz for ^{1}H and 75.48 MHz for ^{13}C. Melting points were determined on a Uniscience Melting Point apparatus without corrections. Si-gel (Merck, 230–400 mesh) was used in the chromatography column. TLC was performed on plates pre-coated with silica gel 60 F_{254} (Merck) and the spots were visualized with (*a*) 10% H_2SO_4 : methanol solution, followed by heating until the appearance of spots and (*b*) *p*-anisaldehyde : sulfuric acid, followed by reheating. Mushroom tyrosinase (6680 U/mg) and L-DOPA were purchased from Sigma Chemical (São Paulo, Brazil). Resorcinol was obtained in analytical grade from Merck (Darmstadt, Germany) and orcinol from Sigma (St. Louis, MO, U.S.A.). Verification of purity grades was based on melting points and NMR spectra. All aqueous solutions were prepared with deionized water. Phosphate buffer (Na_2HPO_4–NaH_2PO_4) at 50.0 mM, pH 6.8, was used in all reactions.

Preparation of Compounds Orsellinic acid (2,4-dihydroxy-6-methylbenzoic acid) (**4**) was obtained by hydrolyzing lecanoric acid (**3**) isolated from a *Parmotrema tinctorum* specimen. Alkyl orsellinates (2,4-dihydroxy-6-methyl benzoates) (**5**–**15**) were prepared by reacting 200 mg of lecanoric acid (**3**) with 50 mL of the corresponding alcohol at 40°C. The compounds were purified by chromatography on a silica column with chloroform and a chloroform : acetone gradient. Details of the experimental procedure and spectral characterization for compounds **1**–**11** can be found in Lopes *et al.*[20]

Pentyl-2,4-dihydroxy-6-methyl benzoate (*n*-pentyl orsellinate) (**12**): ^{1}H-NMR (acetone-d_6) δ: 11.76 (1H, s, ArOH-2), 9.63 (1H, s, ArOH-4), 6.28 (1H, s, ArH-5), 6.24 (1H, s, ArH-3), 4.33 (2H, t, J=7.35 Hz, CH_2-1′), 2.48 (3H, s, $ArCH_3$-8), 1.78 (2H, m, CH_2-2′), 1.42 (2H, m, CH_2-3′-4′), 0.92 (3H, m, CH_3-5′). ^{13}C-NMR (acetone-d_6) δ: 172.5 (C-7), 165.6 (C-4), 162.6 (C-2), 143.5 (C-6), 111.6 (C-5), 104.4 (C-1), 100.8 (C-3), 65.2 (C-1′), 29.1 (C-2′), 28.2 (C-3′), 28.1 (C-4′), 23.7 (C-8), and 13.4 (C-5′). mp 58–59°C.

Hexyl-2,4-dihydroxy-6-methyl benzoate (*n*-hexyl orsellinate) (**13**): ^{1}H-NMR (acetone-d_6) δ: 11.72 (1H, s, ArOH-2),

6.28 (1H, s, ArH-5), 6.23 (1H, s, ArH-3), 4.34 (2H, t, J=7.35 Hz, CH_2-1′), 2.48 (3H, s, $ArCH_3$-8), 1.79 (2H, m, CH_2-2′), 1.47 (2H, m, CH_2-3′) 1.35 (2H, m, CH_2-4′-5′), 0.89 (3H, m, CH_3-6′). ^{13}C-NMR (acetone-d_6) δ: 172.4 (C-7), 166.6 (C-4), 163.1 (C-2), 144.0 (C-6), 112.0 (C-5), 105.0 (C-1), 101.3 (C-3), 65.7 (C-1′), 31.8 (C-2′), 28.9 (C-3′), 26.2 (C-4′), 24.1 (C-8), 22.8 (C-5′), and 13.9 (C-6′). mp 46–48°C.

Octyl-2,4-dihydroxy-6-methyl benzoate (*n*-octyl orsellinate) (**14**): ^{1}H-NMR (acetone-d_6) δ: 11.91 (1H, s, ArOH-2), 6.28 (1H, s, ArH-5), 6.23 (1H, s, ArH-3), 4.33 (2H, t, J=7.35 Hz, CH_2-1′), 2.50 (3H, s, $ArCH_3$-8), 1.77 (2H, m, CH_2-2′), 1.42 (2H, m, CH_2-3′), 1.31 (2H, m, CH_2-4′-7′), 0.88 (3H, m, CH_3-8′). ^{13}C-NMR (acetone-d_6) δ: 171.9 (C-7), 165.4 (C-4), 160.2 (C-2), 144.0 (C-6), 111.3 (C-5), 105.8 (C-1), 101.3 (C-3), 65.6 (C-1′), 31.7 (C-2′), 29.2 (C-3′), 28.6 (C-4′), 26.2 (C-5′), 24.4 (C-8), 22.6 (C-6′-7′), and 14.10 (C-8′). mp 62–63°C.

Hexadecyl-2,4-dihydroxy-6-methyl benzoate (*n*-cetyl orsellinate) (**15**): ^{1}H-NMR (acetone-d_6) δ: 11.86 (1H, s, ArOH-2), 6.27 (1H, s, ArH-5), 6.22 (1H, s, ArH-3), 4.32 (2H, t, J=7.35 Hz, CH_2-1′), 2.50 (3H, s, $ArCH_3$-8), 1.77 (2H, m, CH_2-2′), 1.62 (2H, m, CH_2-3′), 1.25 (2H, m, CH_2-4′-15′), 0.88 (3H, m, CH_3-16′). ^{13}C-NMR (acetone-d_6) δ: 171.8 (C-7), 165.5 (C-4), 160.2 (C-2), 144.0 (C-6), 111.3 (C-5), 105.3 (C-1), 101.3 (C-3), 65.6 (C-1′), 32.0 (C-2′), 29.7 (C-3′), 29.4 (C-4′), 24.4 (C-8), 22.7 (C-5′-15′), and 14.13 (C-16′). mp 84–85°C.

Enzymatic Activity Inhibition of the diphenolase activity of mushroom tyrosinase was evaluated using L-DOPA as the substrate.[25] The test compounds were first dissolved in dimethyl sulfoxide (DMSO) at 10.0 mM for samples **1**–**2** and **4**–**14** and, because of lower solubility, at 3.3 mM for samples **3** and **15**. For analysis of samples **1**–**2** and **4**–**14**, 0.1 mL of sample solution in DMSO was combined with 1.5 mL of sodium phosphate buffer. For samples **3** and **15**, 0.3 mL of sample solution in DMSO was combined with 1.2 mL of the same buffer. Each resulting solution was then mixed with 0.2 mL of L-DOPA (3.5 mM) and 0.2 mL of tyrosinase (10 μg/mL). The mixtures were homogenized and incubated for 5 min at 30°C. Absorbances were measured at 475 nm to monitor the formation of dopachrome (ε=3700 $M^{-1}cm^{-1}$) using a Spectrum 220 spectrophotometer. Tyrosinase activity was calculated by comparing with a control reaction:

$$Tyrosinase\ activity\% = \left[\frac{(A_{\text{sample}} - A_{\text{control}})}{B_{\text{control}}}\right] \times 100\%$$

where A_{sample} is the absorbance of the sample reaction (containing all reagents and the test compound), A_{control} is the absorbance of the test compound, and B_{control} is the absorbance of the control reaction (containing all reagents, but no test compound). For inhibitors, tyrosinase activity <100%; for activators, tyrosinase activity >100%. All tests were performed in triplicate.

n-Octyl orsellinate was subjected to kinetic analysis to determine inhibition type and the respective constant. The Lineweaver–Burk equation in double-reciprocal form and secondary replots were applied.[25] The inhibition constant of the uncompetitive inhibitor (KESI) was determined using the graphical method.[26]

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Conflict of Interest The authors declare no conflict of interest.

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