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Synthesis and biological evaluation of a novel series of bis-salicylaldehydes as mushroom tyrosinase inhibitors

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ABSTRACT

A series of novel bis-salicylaldehydes were synthesised and evaluated as tyrosinase inhibitors using a tyrosinase-dependent L-DOPA oxidation assay. The bis-salicylaldehydes exhibited greater inhibitory activity than salicylaldehyde. Our data suggests that these novel compounds may serve as a structural template for the design and development of novel tyrosinase inhibitors.

During the past decade, benzaldehydes from natural and synthetic sources as well as other derivatives, such as *trans*-cinnamaldehyde,[1] 2-hydroxy-4-methoxybenzaldehyde, and anisaldehyde,[2] have been isolated and evaluated as mushroom tyrosinase inhibitors. Tyrosinase (monophenol or *o*-diphenol, oxygen oxidoreductase, EC 1.14.18.1, syn. polyphenol oxidase) is a copper-containing enzyme that is present in micro-organisms, animals and plants. Tyrosinase catalyses the hydroxylation of monophenols and oxidation of *o*-diphenols to *o*-quinones using molecular oxygen; *o*-quinones spontaneously polymerise to form dark macromolecular pigments, such as melanin.[3] In mammals, L-tyrosine is converted to L-DOPA, oxidised to dopaquinone and ultimately transformed into melanin. This metabolic pathway is very important, and melanin synthesis is altered in many disease states. Melanogenesis inhibitors are clinically used for the treatment of skin disorders associated with hyperpigmentation.[4] These chemicals are also used as cosmetics for skin whitening.[5] Melanin pigments are found in the mammalian brain, and tyrosinase activity is linked to neurodegeneration associated with Parkinson[6,7] and other degenerative diseases. Moreover, melanin formation is also considered to be deleterious to the colour quality of fruit and vegetables. Inhibition of this enzyme may be useful to prevent browning of produce.[8] Furthermore, tyrosinase is important for the insect moulting process[9] and adhesion in marine organisms.[10,11]

Interest in benzaldehydes was generated after review of the literature.[12–14] Aldehyde reacts with biologically important nucleophilic groups, such as sulphydryl, amino, and hydroxyl. It has been suggested that its inhibitory effect is due to the formation of a Schiff base with the primary amino group.[15] An electron-donating group, such as isopropyl at the *para* position in cuminaldehyde (4-isopropylbenzaldehyde) (IC_{50} of 0.05 mM) seems to contribute not only to the inductive effect but also to the stability of the binding sites in the enzyme. It appears that the isopropyl group is better embraced by the hydrophobic protein pocket compared to the methoxy group in anisaldehyde (IC_{50} of 0.32 mM). Specifically, the presence of a hydrophobic electron donor group at the *para* position in benzaldehyde elicits greater inhibitory activity. The addition of a methoxy group at the *meta* or *para* position of salicylaldehyde shows different effects. Despite their close structural similarity, 2-hydroxy-4-methoxybenzaldehyde induced greater inhibitory activity (IC_{50} of 0.03 mM) than 2-hydroxy 3-methoxybenzaldehyde (IC_{50} of 26 mM). The introduction of a strong electron-donating methoxy group in the *para* position stabilises the Schiff base and increases the inhibitory activity.[12] It is known that the Schiff base is largely governed by those factors affecting the stability of the carbon–nitrogen double bond. In addition, the ortho hydroxybenzaldehyde moiety in these compounds forms a nearly six-membered ring through intramolecular hydrogen bonding and produces a stable chelate structure.

In this Letter, we investigated the inhibitory activity of various chemical group substitutions in the benzaldehyde ring of bis-salicylaldehydes. We report the synthesis and evaluation of five

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5,5′-methylene-bis-salicylaldehyde compounds to establish a structure–activity relationship for these compounds. The kinetic behaviour of mushroom tyrosinase-dependent oxidation of L-DOPA was determined in the presence of various concentrations of compound. In the literature, the IC_{50} value for tyrosinase inhibitors often varies due to different assay conditions, such as substrate concentration, incubation time, and batches of commercial tyrosinase. Fortunately, in most studies a well-characterised tyrosinase inhibitor, such as kojic acid, is often used as a positive standard.

The preparation of bis-salicylaldehydes **2–4**[16–20] was performed by the reaction of salicylaldehyde **1a–c** with a solution of paraformaldehyde in a mixture of glacial acetic acid and sulphuric acid under nitrogen atmosphere. The resulting solution was heated to 90–95 °C with stirring for 24 h (Scheme 1). Compounds **2**,[16,17] **3**[18,19] and **4**[20] were obtained in yields of 48%, 54% and 58%, respectively. The reaction mixture was purified by column chromatography, using dichloromethane/petroleum ether at a ratio of 6:1 as eluent. *p*-Methoxy derivatives **3** and **4** were hydrolysed with iodidric acid in the presence of acid acetic and acetic anhydride and refluxed for 3 h.[21] The solvent was then removed under a vacuum, and the residue was purified by crystallisation in acetone to give **5**[22,23] and **6**[24] in 51% and 46% yields, respectively (Scheme 1).

The effects of compounds **2–6** on tyrosinase activity was determined using the L-DOPA oxidation assay.[25] Enzyme activity was directly related to inhibitor molecule concentration; however, as shown in Figure 1 (compound **2**), it was not completely suppressed at any concentration tested. Each compound investigated displayed greater inhibitory activity than the reference compound salicylaldehyde (see Table 1). Compound **2** displayed the greatest inhibition, with an IC_{50} value of 0.074 mM. This value, determined using the same experimental conditions, was 10-fold higher than for benzaldehyde. The inhibitory mechanism of this compound was determined using a Lineweaver–Burk double reciprocal plot. Figure 2 shows the double-reciprocal plot of enzyme activity in the presence of the compound **2**. The data, displayed as a plot of $1/V$ versus $1/[S]$, gave three straight lines with different slopes and a horizontal line that intersected at the same point. With an increase in compound concentration, the V_{max} value decreased, whereas the K_m value remained the same, suggesting that this compound is a non-competitive inhibitor of tyrosinase. The inhibition constant of this compound (K_I of 0.048 mM) was determined by plotting the intercept values versus the concentration of the corresponding compound, as shown in Figure 2. Compounds **3** and **5** showed a slight increase in tyrosinase inhibitory activity with respect to salicylaldehyde, but caused less inhibition than compound **2**. When the methoxy group of compound **3** was replaced with a hydroxy group, as in compound **5**, the IC_{50} value decreased slightly. The introduction of a methoxy group in the *para* position of compound **2** formed compound **4** and increased the inhibitory activity sixfold compared to compound **3**, an isomer of compound **4**. The introduction of a hydroxy group, but not a methoxy group, in the *para* position (**6**) led to better inhibitory activity. The IC_{50} value obtained for this compound was comparable to that of compound **2** (see Table 1).

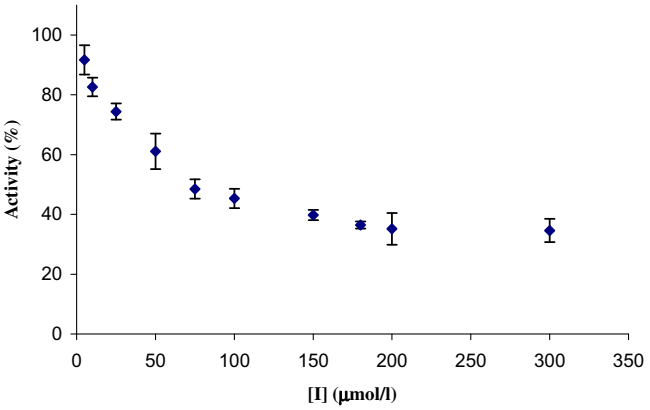
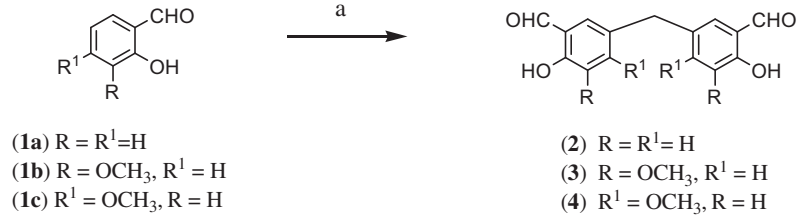


Figure 1. Inhibition of compound **2** on the mushroom tyrosinase.

Table 1
The inhibitory effects of salicylaldehyde and its derivatives (**2–6**) on mushroom tyrosinase activity

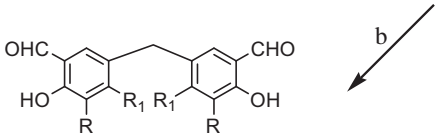
Compound tested	IC_{50} (mM) (L-DOPA 0.35 mM)
Salicylaldehyde	0.833 ± 0.1600
2	0.074 ± 0.0342
3	0.695 ± 0.1651
4	0.117 ± 0.0002
5	0.532 ± 0.2012
6	0.076 ± 0.0392
Kojic acid	$8.33 \times 10^{-3} \pm 1.655 \times 10^{-3}$

In our study, we report the synthesis of a novel series of bis-salicylaldehydes and their effects on the diphenolase activity of mushroom tyrosinase to oxidise L-DOPA. The inhibitory activity of each compound was determined by measuring the IC_{50}. The experimental results reported in this paper show for the first time



(**1a**) R = R^1=H
(**1b**) R = OCH$_3$, R^1 = H
(**1c**) R^1 = OCH$_3$, R = H

(**2**) R = R^1= H
(**3**) R = OCH$_3$, R^1 = H
(**4**) R^1 = OCH$_3$, R = H



(**5**) R = OH, R^1 = H
(**6**) R^1 = OH, R = H

Scheme 1. General synthetic route to obtain compounds **2–6**. Reagents and conditions: (a) HCHO, AcOH, H_2SO_4, 90 °C, 24 h; (b) HI/AcOH, 0 °C to rt, 3 h.

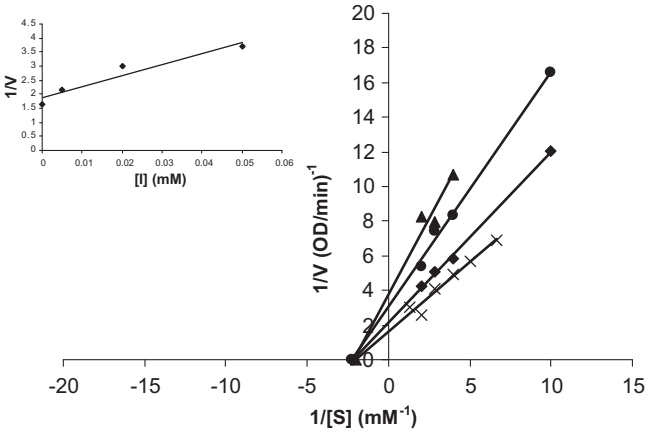


Figure 2. Lineweaver–Burk plots for inhibition of compound **2** on mushroom tyrosinase for catalysis of L-DOPA. Concentrations of **2** were 0, 0.005 mM, 0.02 mM, 0.05 mM, respectively. The inset represent the secondary plot of $1/V_{max}$ versus concentration of compound **2**, to determine the inhibition constant (K_i).

that bis-salicylaldehyde compounds act as better inhibitors of mushroom tyrosinase than salicylaldehyde. Moreover, we determined that compound (**2**), the most effective inhibitor, acts as a non-competitive inhibitor. We determined the effects of different chemical groups on the inhibitory activity of these compounds. First, the joining of two benzaldehyde rings facilitated the inhibitory effects on the diphenolase activity of mushroom tyrosinase. Second, the position of substitutions on the phenyl rings and the type of substitutions critically affected the inhibitory activity. Indeed, the substituents introduced in the *meta* position on the phenyl rings may have hindered docking of the inhibitor to tyrosinase, whereas substitutions in the *para* position did not affect the enzyme–inhibitor interaction. Furthermore, when the –OH groups were in the *ortho* position of each benzaldehyde ring each compound exhibited remarkable tyrosinase inhibition.

These results suggest that these novel compounds may serve as structural templates for the design and development of novel tyrosinase inhibitors.

Acknowledgments

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17. *5,5′-Methylene-bis-salicylaldehyde* (***2***). Purified by chromatography using 6:1 dichloromethane/petroleum ether as the eluent. Mp. 141–142 °C (Ref. 14 142–143 °C). ^{1}H NMR ($CDCl_3$) δ (ppm) 3.96 (s, 2H, $-CH_2$), 6.95 (d, 2H, aromatic), 7.26 (s, 2H, aromatic), 7.34 (d, 2H, aromatic), 9.85 (s, 2H, CHO), 10.91 (s, 2H, OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 39.35, 117.92, 120.41, 131.90, 133.15, 137.52, 160.14, 196.34. MS *m*/*z* (%): 256 (100), 227 (67), 209 (17), 199 (25), 181 (75), 152 (12), 135 (22), 107 (15), 77 (22).
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19. *3,3′-Dimethoxy-5,5′-methylene-bis-salicylaldehyde* (***3***). Purified by chromatography using 6:1 dichloromethane/petroleum ether as the eluent. Mp. 157–158 °C (Ref. 16156–158 °C). ^{1}H NMR ($CDCl_3$) δ (ppm): 3.81 (s, 6H, $-OCH_3$), 3.96 (s, 2H, $-CH_2$), 6.85 (s, 2H, aromatic), 6.87 (s, 2H, aromatic), 9.81 (s, 2H, –CHO), 10.93 (s, 2H, –OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 40.44, 56.37, 113.32, 123.84, 127,58, 133.02, 150, 40, 196.38. MS *m*/*z* (%): 316 (100), 288 (12), 270 (5), 165 (8).
20. *4,4′-Dimethoxy-5,5′-methylene-bis-salicylaldehyde* (***4***): This compound was obtained with a yield of 58%: Mp.: 185–187 °C. IR (KBr): 1670 (–C═O) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 3.81 (s, 6H, $-OCH_3$), 3.99 (s, 2H, $-CH_2$), 7.50 (d, 2H, J = 8.7), 7.30 (d, 2H, J = 8.7), 9.67 (s, 2H, CHO), 11.44 (s, 2H, OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 29.70, 56.04, 103.22, 132.14, 133.7, 134.5, 161.49, 164.77, 194.75. MS *m*/*z* (%): 316 (100), 288 (9), 270 (5), 165 (80), 151 (17), 137 (12), 77 (2). Anal. Calcd for $C_{17}H_{16}O_6$: C, 64.55; H, 5.10. Found: C, 64.7; H, 5.22.
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23. *3,3′-Dihydroxy-5,5′-methylene-bis-salicylaldehyde* (***5***). It was obtained with a yield of 51%: mp: 190–192 °C (Ref.21 188–189 °C, yield 41%).
24. *4,4′-Dihydroxy-5,5′-methylene-bis-salicylaldehyde* (***6***): This compound was obtained with a yield of 46%: Mp: 193–195 °C. IR (KBr): 1650 (–C═O) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ (ppm): 3.92 (s, 2H, $-CH_2$), 7.30 (d, 2H, J = 9.0), 7.65 (d, 2H, J = 9.0), 9.81 (s, 2H, CHO), 10.81 (s, 2H, OH), 11.63 (s, 2H,OH). ^{13}C NMR (DMSO-d_6) δ (ppm): 43.0, 120.0, 122.4, 135.0, 137.5, 140.6, 163.2, 198.0. MS *m*/*z* (%): 288 (100), 260 (17), 151 (91), 137 (32), 77 (5). Anal. Calcd for $C_{15}H_{12}O_6$: C, 62.50; H, 4.20. Found: C, 62.54; H, 4.22.
25. Pre-incubation with the enzyme consisted of a 1/15 M phosphoric acid buffer solution (pH 6.8, 1.8 mL), an aqueous solution of mushroom tyrosinase (1000 U/mL, Sigma Chemical Co., 0.1 mL) and DMSO (0.1 mL) with or without the sample. The mixture was incubated at 25 °C for 10 min. Then, a 1.05 mM of L-3,4-dihydroxyphenylalanine (DOPA) solution (1 mL) was added and the reaction was monitored at 475 nm for 5 min. The percent inhibition of tyrosinase activity was calculated as: inhibition (%) = $(A - B)/A \times 100$, where A represents the difference in the absorbance of control sample between 0.5 and 1.0 min, and B represents the difference in absorbance of the test sample between 0.5 and 1.0 min. The IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of dose–response curves. The activity of mushroom tyrosinase was determinate by spectrophotometric techniques (Varian Cary 50). Kojic acid was used as a reference tyrosinase inhibitor.