Journal Pre-proofs

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PII: S0960-894X(19)30680-8
DOI: https://doi.org/10.1016/j.bmcl.2019.126722
Reference: BMCL 126722

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 17 June 2019
Revised Date: 26 September 2019
Accepted Date: 27 September 2019

Please cite this article as: Nazir, Y., Saeed, A., Rafiq, M., Afzal, S., Ali, A., Latif, M., Zuegg, J., Hussein, W.M., Fercher, C., Barnard, R.T., Cooper, M.A., Blaskovich, M.A.T., Ashraf, Z., Ziora, Z.M., Hydroxyl substituted benzoic acid/cinnamic acid derivatives: tyrosinase inhibitory kinetics, anti-melanogenic activity and molecular docking studies, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.126722

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Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Hydroxyl substituted benzoic acid/cinnamic acid derivatives: tyrosinase inhibitory kinetics, anti-melanogenic activity and molecular docking studies.

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Methoxy phenol,
tyrosinase inhibitors,
kinetic mechanism,
melanin quantification,
molecular docking studies

ABSTRACT

The inhibition of tyrosinase is an established strategy for treating hyperpigmentation. Our previous findings demonstrated that cinnamic acid and benzoic acid scaffolds can be effective tyrosinase inhibitors with low toxicity. The hydroxyl substituted benzoic and cinnamic acid moieties of these precursors were incorporated into new chemotypes that displayed *in vitro* inhibitory effect against mushroom tyrosinase. The most active compound, (2-(3-methoxyphenoxy)-2-oxoethyl (E)-3-(4-hydroxyphenyl) acrylate) **6c**, inhibited tyrosinase with an IC_{50} of 5.7 µM, while (2-(3-methoxyphenoxy)-2-oxoethyl 2, 4-dihydroxybenzoate) **4d** had an IC_{50} of 23.8 µM. In comparison, the positive control, kojic acid showed tyrosinase inhibition with an IC_{50} =16.7 µM. Analysis of enzyme kinetics revealed that **6c** and **4d** displayed noncompetitive reversible inhibition of the second tyrosinase enzymatic reaction with K_i values of 11 µM and 130 µM respectively. *In silico* docking studies with mushroom tyrosinase (PDB ID 2Y9X) predicted possible binding modes in the catalytic site for these active compounds. The phenolic *para*-hydroxy group of the most active compound **6c** is predicted to interact with the catalytic site Cu^{++} ion. The methoxy part of this compound is predicted to form a hydrogen bond with Arg 268. Compound **6c** had no observable toxic effects on cell morphology or cell viability at the highest tested concentration of 91.4 µM. When dosed at 91.4 µM onto B16F10 melanoma cells *in vitro* **6c** showed anti-melanogenic effects equivalent to kojic acid at 880 µM. **6c** displayed no PAINS (pan-assay interference compounds) alerts. Our results show that compound **6c** is a more potent tyrosinase inhibitor than kojic acid and is a candidate for further development. Our exposition of the details of the interactions between **6c** and the catalytic pocket of tyrosinase provides a basis for rational design of additional potent inhibitors of tyrosinase, built on the cinnamic acid scaffold.

Tyrosinase is widely distributed in nature, from bacteria to plants and humans. It catalyses the *o*-hydroxylation of monophenols to catechols and the subsequent two-electron oxidation to quinones [1]. Its physiological function is the conversion of tyrosine into dopaquinone, the first step of melanin biosynthesis in specialized organelles called melanosomes [2-3]. The non-essential aromatic amino acid L-tyrosine serves as precursor to melanin biosynthesis [4]. L-tyrosine and L-DOPA (or phosphorylated isomers of L-DOPA) are key molecules in regulation of the melanin synthesis [5-7]. The distribution patterns of melanin in keratinocytes determine the color of human skin [8-9]. Melanogenesis is modulated by several factors such as UV exposure, α-melanocyte-stimulating hormone, melanocortin-1 receptor and agouti-related protein [10-11]. The process of melanogenesis represents a potential cellular hazard and is confined to special melanosomes in melanocytes, which synthesize pigments and transfer them to recipient cells [12]. Melanoma is a type of skin cancer that arises from the aberrant proliferation of melanocytes [13-14].

Malignant melanocytes tend to exhibit up-regulated melanogenesis and defective melanosomes. Therefore, controlling a tyrosinase-dependent mechanism of melanogenesis might be the basis for a potential anti-melanoma therapy. Specific tyrosinase inhibitors such as kojic

acid, arbutin, azelaic acid, hydroquinone and phenol have been tested in pharmaceutical formulations for their ability to block

overproduction of melanin [15]. However, only kojic acid has shown a slight inhibitory effect against pigmentation within melanocytes [16].

Currently available tyrosinase inhibitors possess undesirable side effects such as carcinogenicity and low clinical efficacy [17]. Therefore, it is of great interest for both medical and cosmetic applications to synthesize novel inhibitors. Recently (2019) Wakana Ishioka et al. described resorcinol derivatives, which are used as active ingredients in food preservatives and functional cosmetics, as potent tyrosinase inhibitors [18]. In 2018 Sultan Ullah et al. reported that cinnamamide derivatives showed much higher inhibitory effect than kojic acid on mushroom tyrosinase and melanin production in B16F10 murine skin melanoma cells, without cytotoxicity at the inhibitory doses [19].

The methoxy phenol moiety commonly found in natural phenolic antioxidants is responsible for a variety of biological activities such as carbonic anhydrase inhibitory activity [20], anti-inflammatory activity [21] and the scavenging of free radicals [22-25]. 3-methoxyphenol has been reported as a depigmenting and therapeutic agent for melanoma therapy [26]. However, the tyrosinase inhibitory activities of compounds possessing a methoxy phenol moiety have only rarely been discussed in the literature [27]. Menezes et al. 2011 and Miliovsky et al. 2013 reported that cinnamic acid and benzoic acid analogues possessing a substituted phenyl ring exhibited antioxidant and tyrosinase inhibitory activity [28-29]. Cinnamic acid has been reported to have extensive physiological actions including tyrosinase inhibitory activity [30].

Our laboratory has synthesized a variety of phenolic compounds, including thymol analogues, vanillin and umbelliferone derivatives (Fig 1) [31-34] that mimick the chemical structure of tyrosinase's natural substrates, L-DOPA and L-tyrosine. We have evaluated their tyrosinase inhibitory activity with a view to discovering potent tyrosinase inhibitors that bind to the active site of tyrosinase more strongly than the natural substrates. From these studies, we concluded that compounds with a phenolic moiety can exhibit potent tyrosinase inhibitory activity. In our studies on tyrosinase inhibitors we have synthesized hydroxyl substituted benzoic and cinnamic acid derivatives to explore their tyrosinase inhibitory activity and to understand how these compounds interact with the catalytic center of the protein with a view to engineering more potent compounds.

The difficulty of isolating pure and homogeneous human tyrosinase from endogenous sources has hindered the studies. Due to the absence of a high-resolution crystal structure of the human enzyme and the fact that both proteins share high sequence similarity in their active core region, mushroom tyrosinase has frequently served as a model to assess the inhibitory activity of compounds [35].

Previous work by Abbas et al. (2017) has validated this approach by showing that the potency of inhibition of mushroom tyrosinase follows the trend in potency against human tyrosinase [36]. Therefore, the initial screening of the synthesized compounds as anti-melanogenic agents was carried out using mushroom tyrosinase. The *in vitro* mushroom tyrosinase inhibitory activity of the target compounds with different hydroxy substituted benzoic acids **(4a-e)** and cinnamic acids **(6a-c)** were evaluated both experimentally and *in silico*.

The *in-silico* molecular docking of all the synthesized compounds was performed against the mushroom tyrosinase crystal structure (PDB ID: 2Y9X) to ascertain the role of different functionalities in the formation of a ligand-protein complex. All of these compounds **4a-e** and **6a-c** passed the PAINS (pan-assay interference compounds) filter [37], with the exception of **4c**. The most potent derivative, **6c,** was selected for further characterization of its effects on cellular tyrosinase inhibition, cell toxicity, cell viability and melanin biosynthesis in B16F10 melanoma cells.

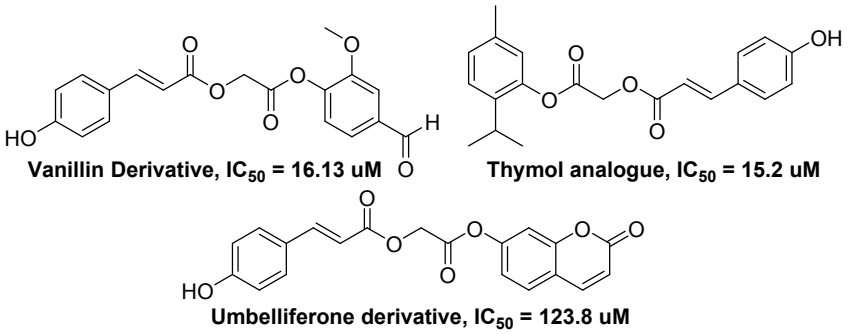
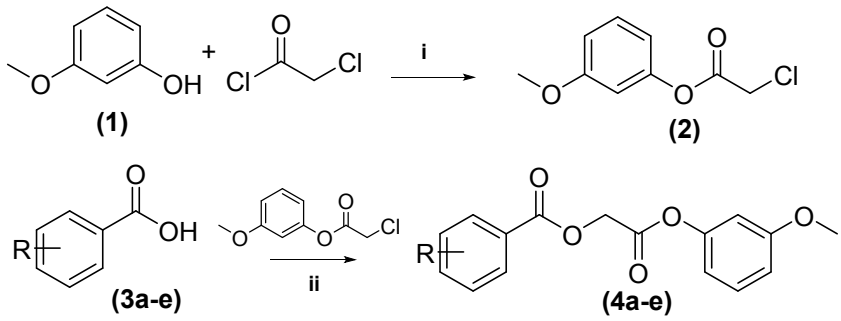
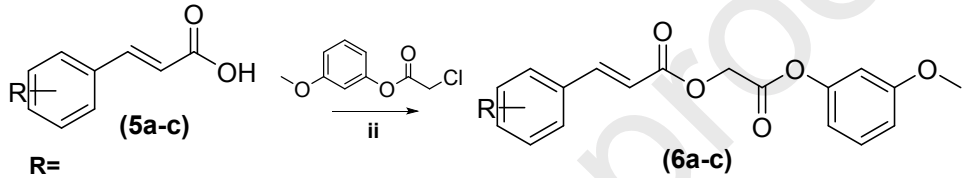


Figure 1. Potent inhibitors of mushroom tyrosinase from previous studies.



R=
a=3-OH, **b**= 4-OH, **c**= 3,4-di-OH, **d**= 2,4-di-OH, **e**= 3,5-di-OH

Scheme 1. Synthesis of compounds **(4a-e)**, Reagents and conditions,
(i) CH_2Cl_2/ $(C_2H_5)_3N$, 0_-5^oC, stirring for 5 hrs, **(ii)** DMF/ $(C_2H_5)_3N$/KI, r. t. stirring for 24 hrs.



R=
a= -H, **b=** 2-OH**, c=** 4-OH

Scheme 2. Synthesis of compounds **(6a-c)**, **(ii)** DMF/ $(C_2H_5)_3N$/KI, r. t. stirring for 24 hrs.

The *in-vitro* mushroom tyrosinase inhibitory activity of all the synthesized compounds **(4a-e)** and **(6a-c)** were determined by following the previously described method [38], with slight modifications as detailed in the supplementary material. The synthesized compounds **(6a)**, **(6b)** and **(6c)** are all cinnamate esters but differ regarding the substitution pattern on the cinnamate phenyl ring. Compound **(6a)** possesses an unsubstituted cinnamic acid component while compound **(6b)** and **(6c)** possess 2-hydroxy and 4-hydroxy substituted cinnamic acid, respectively. The tyrosinase inhibitory activity of 4-hydroxy cinnamic acid is already reported [39], its incorporation as an ester in compound **6c** results in an increase in the activity. The replacement of hydrogen from the para position of the cinnamic acid phenyl ring in compound **(6a)** with a hydroxyl group **(6c)** led to a significant increase in tyrosinase inhibitory activity (Table 1). The compound **(6a)** (IC_{50} 39.4 µM) was 7-fold less active while **(6b)** (IC_{50} 172.8 µM) was 30-fold less active than **(6c)**. It has already been well established that phenolic hydroxyl plays a vital role in tyrosinase inhibitory activity as the natural substrates tyrosine and L-DOPA also bear the phenolic hydroxyls [40]. Here, we report that the para-hydroxyl group of **(6c)** appears to play a similar role in tyrosinase inhibitory activity.

Table 1. Tyrosinase inhibitory activity, substitution pattern, kinetics and % yield of compounds **(4a-e)** and **(6a-c)**

Compounds	R^1	R^2	Mushroom tyrosinase IC_{50} (µM)	K_i(µM)	Yield (%)
4a	3-OH		314.5 ± 12.6	n.d.*	81
4b	4-OH		337.7 ± 17.2	-	79
4c	3,4-di-OH		298.7 ± 15.6	-	78
4d	2,4-di-OH		23.8 ± 3.7	130	73
4e	3,5-di-OH		192.7 ± 11.6	n.d.*	80
6a		-H	39.4 ± 4.5	-	82
6b		2-OH	172.8 ± 8.8	-	81
6c		4-OH	5.7 ± 0.3	11	81
Kojic Acid			16.7 ± 2.8	n.d.*	

***n.d.= not determined**

Kinetic analysis was conducted with compounds **(4d)** and **(6c)** using the mushroom tyrosinase enzyme and L-DOPA as substrate [41]. The (1/V versus 1/ [S]) in Fig. 2, which showed for both compounds a slight preference of non-competitive inhibition over competitive inhibition. The plots of 1/V versus 1/ [S] gave a family of straight lines with different slopes. The analysis showed that V_{max} decreased with changing K_m in the presence of increasing concentrations of compound**.** This result indicates that both compounds **(4d)** and **(6c)** bind not only to the free enzyme, but also to some degree to the enzyme-substrate complex [42]. Similarly, analyzing the kinetic inhibition using a Dixon plot (Fig. 2) indicates a slight preference for noncompetitive inhibition over competitive inhibition, with K_i values of 130 µM and 11 µM, for **(4d)** and **(6c)** respectively. Moreover, the inhibitory mechanism of mushroom tyrosinase by compound **(6c)** on the oxidation of L-DOPA was studied. The plots of the remaining enzyme activity versus the concentration of enzyme (4, 6, 8 and 10 µg/mL, respectively) in the presence of different concentrations of compound **(6c)** for the catalysis of L-DOPA gave a series of straight lines (Fig.3), which all intersected at the same point on X-axis, indicating that compound **(6c)** is a reversible inhibitor of tyrosinase [43].

type of inhibition was determined using Lineweaver-Burk plots

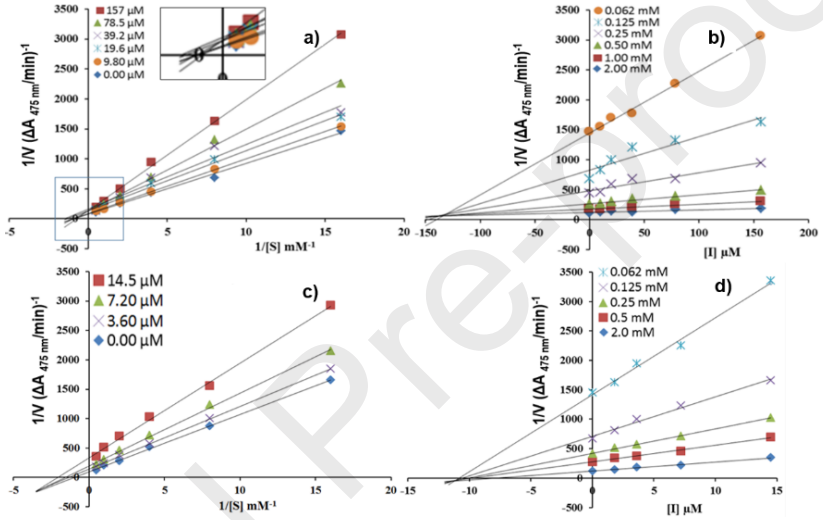


Figure 2. Kinetic analysis of **(4d** and **6c)** analyzing type of inhibition. **a,c)** Lineweaver-Burk plot **b,d)** Dixon plots. The concentration of compound **(4d)** was 0.00 to 157 µM and that of compound **6c** was 0.00 to 14.5 µM and the concentration of L-DOPA was 0.062 to 2.00 mM, determining a slight preference for a non-competitive inhibition over competitive inhibition.

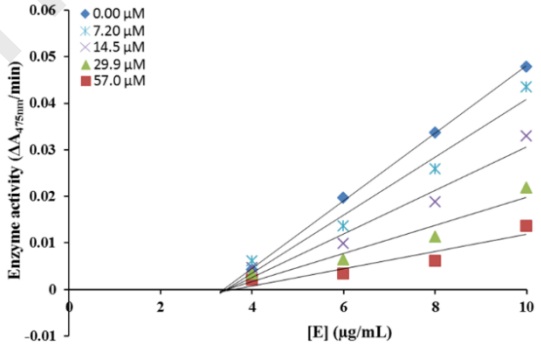


Figure 3. Relationship between the catalytic activity of tyrosinase at different concentrations of the enzyme and compound **(6c)**.The lines intersecting at the same point on X-axis indicates that 6c is a reversible inhibitor.

A cell viability assay in B16F10 melanoma cells using MTT [3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was applied to assess potential cytotoxic effects of **(6c)** [44]. Cell viability was found to be higher than 95% and 99% when cells were incubated with 40 and 30 µg/mL, respectively (Figure 4). Consequently, we selected 30 µg/mL as the highest concentration for further investigation as this

concentration had no considerable cytotoxic effects on cell morphology (Figure S1) and cell viability.

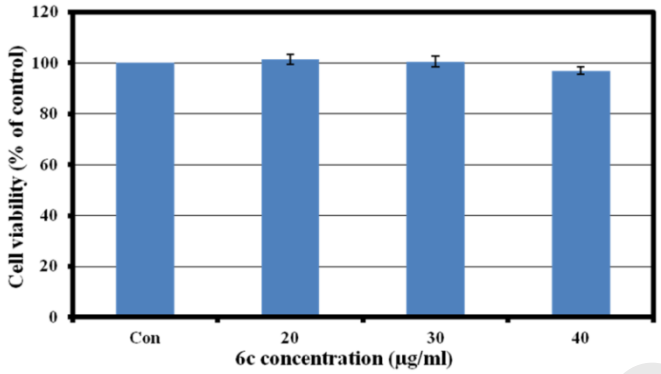


Figure 4. Viability of **B16F10** cells, treated with **(6c)** at different concentrations. Data is normalized against untreated samples (Con), displaying means and standard deviation. No significance by Student's t-tests to Control.

The inhibitory effects on tyrosinase and cellular melanogenesis were also evaluated in B16F10 cells, normalizing results to treatment with kojic acid. The effect of compound **(6c)** on the melanin content in B16F10 cells was evaluated by treating the cells with **(6c)** in the presence of 100 µM α-MSH at different concentrations (20, 25 and 30 µg/mL). The inhibitory effects of **(6c)** are more potent than that of kojic acid as compound **(6c)** showed same inhibition at 30 µg/mL as compared to kojic acid at 125 µg/mL (**Fig. 5a**). Within the melanogenesis cascade of enzymatic reactions, tyrosinase acts as the rate-limiting enzyme [45-46]. To analyze the inhibition mechanism of melanin synthesis by **(6c)**, we examined the inhibitory effect of **(6c)** on cellular tyrosinase activity. We found significant intracellular tyrosinase inhibition at 25 and 30 µg/mL concentration of **(6c)** in a dose-dependent manner **(Fig. 5b)**.

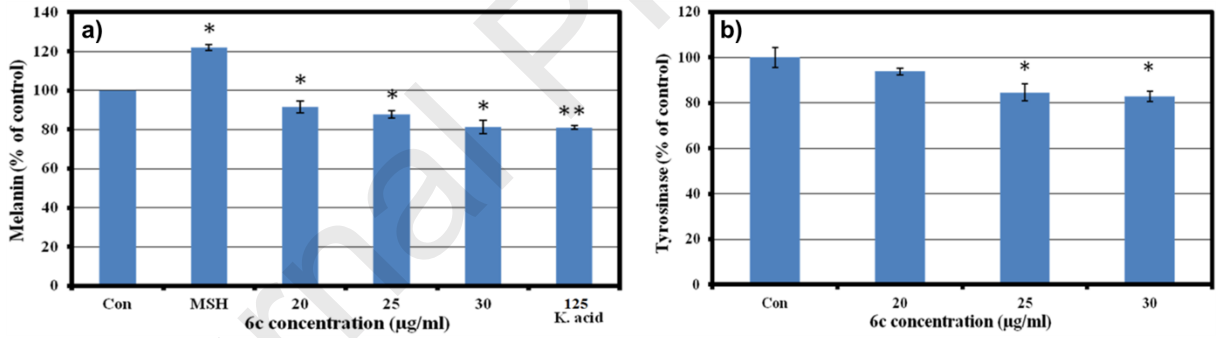


Figure 5. **(a)** Melanin inhibition in B16F10 cells by **(6c)** at different concentrations, with MSH (100 µM) as a known stimulant of melanin production and kojic acid (880 µM) as a known inhibitor. Intracellular melanin was monitored by absorbance at 405 nm. **(b)** Inhibition of tyrosinase activity in B16F10 cells by **(6c)** at different concentrations. Cellular murine tyrosinase activity was monitored by transformation of L-DOPA into dopachrome by absorbance at 490 nm. Data is normalized against untreated samples (Con), displaying means and standard deviation, and significance by Student's t-tests compared to control: *p<0.05, **p<0.005.

Computational docking studies were performed to predict the most favorable binding mode of **(6c)** with 2Y9X inside the binding pocket of the mushroom tyrosinase with proper orientation in term of docking score –5.934 Kcal/ mol[47]. Such lower values indicate good fitness of the compound in the binding pocket of the protein and stable **6c**-protein interaction (**Fig.6**). The selected pose of the most active compound **(6c)** was visualized in order to determine the amino acids of tyrosinase that are involved in binding of the ligand. For the most active compound (**Fig. 6**), the para hydroxyphenol moiety shows consistent interactions between the hydroxyl and the active site Cu ions. The "tail" moiety of this compound occupies a distinct area of the binding site close to Arg 268, picking up an H-bond interaction between the oxygen of methoxy phenol and the hydrogen of Arg 268. While the docking poses have some degree of uncertainty, they give direction to modifications that could improve the activity of the compounds, and to the bonding features that will need to be retained in new compounds based on this framework.

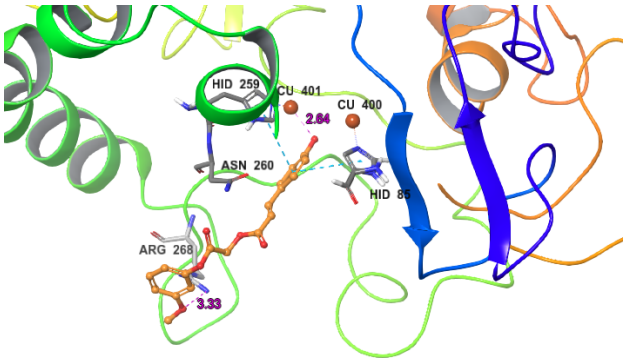


Figure 6. Ball and stick representation of the docking pose of the highly ranked pose for most active compound **6c** in the binding site of mushroom tyrosinase (2Y9X). Residues, in stick representation, that forming the binding site of the enzyme are labeled, Cu^{2+} ions are shown in brown and labeled.

In summary, we have identified *2-(3-methoxyphenoxy)-2-oxoethyl (2E)-3-(4-hydroxyphenyl) prop-2-enoate* **(6c)** as a novel anti-tyrosinase compound that is more potent than the reference compound kojic acid. It appears, based on kinetic analysis, that the mode of inhibition by **6c** is noncompetitive inhibition. This leads us to suggest that the bulky side chains, distant from the metallic center of the binding site are contributing to the non-competitive component of inhibition. The compound appears to reduce α-MSH stimulated melanogenesis through noncompetitive and reversible inhibition of tyrosinase *in vitro,* having a K_i value of 11 μM. Docking studies suggested possible binding poses and interactions for **6c** in the active site of mushroom tyrosinase. The absence of cytotoxic effects of **(6c)** at the concentrations that inhibited melanogenesis in B16F10 cells, supports the suggestion that the suppression of melanogenesis by compound **(6c)** can be attributed to the specific inhibition of tyrosinase. The melanin inhibition results (Fig. 5a) demonstrated that **6c** has de-pigmenting activity at a lower dose than kojic acid (91.4 μM compared to 880 μM) in the B16F10 cell system. It is evident from our *in vitro* results that the presence of a hydroxyl-substituted cinnamate ester scaffold conferred more potent tyrosinase inhibitory activity than is possessed by kojic acid. The compound **(6c)** by virtue of its anti-melanogenic activity, is a promising regulator of melanogenic activity and is a candidate for the further development of agents that have inhibitory effects on tyrosinase and melanin biosynthesis. Our exposition of the interaction between 6c and the catalytic pocket of tyrosinase provides a basis for rational design of additional potent inhibitors of tyrosinase that are built on the cinnamic acid scaffold.

Conflicts of Interest: The authors declare no conflict of interest.

Acknowledgements MAC is a NHMRC principal research fellow (APP1059354) and also holds a fractional professorial research fellow appointment at the University of Queensland, with his remaining time as CEO of Inflazome Ltd, a company developing drugs to address clinical unmet needs in inflammatory disease. MATB is supported in part by Wellcome Trust Strategic Grant WT1104797/Z/14/Z and NHMRC Development grant APP1113719. JZG is supported in part by Wellcome Trust Strategic Grant WT1104797/Z/14/Z. RTB is a chief investigator in the ARC Training Centre for Biopharmaceutical Innovation and received funding from the University of Queensland. YN thanks the Higher Education Commission HEC, Pakistan for providing funding to undertake research at IMB-UQ, Cooper group, Australia.

Appendix A. Supplementary data

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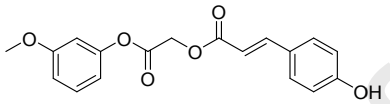
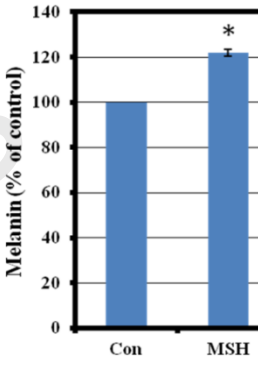
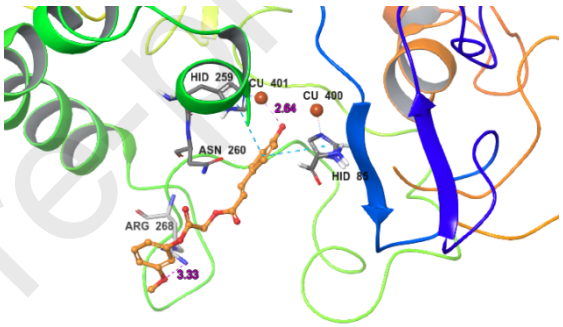


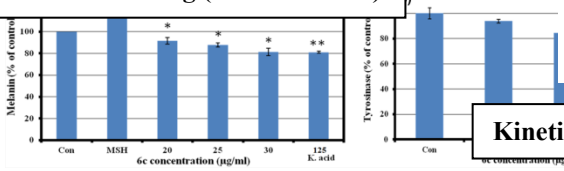
Tyrosinase Inhibitor IC_{50}= 5.7 uM





Melanin inhibi

Molecular docking (PDB ID 2Y9X)



Kinetic analysis of th

Table 1. Tyrosinase inhibitory activity, substitution pattern, kinetics and % yield of compounds **(4a-e)** and **(6a-c)**

Compounds	R^1	R^2	Mushroom tyrosinase IC_{50} (μM)	K_i(μM)	Yi
4a	3-OH		314.5 ± 12.6	n.d.*	
4b	4-OH		337.7 ± 17.2	-	
4c	3,4-di-OH		298.7 ± 15.6	-	
4d	2,4-di-OH		23.8 ± 3.7	130	
4e	3,5-di-OH		192.7 ± 11.6	n.d.*	
6a		-H	39.4 ± 4.5	-	
6b		2-OH	172.8 ± 8.8	-	
6c		4-OH	5.7 ± 0.3	11	
Kojic Acid			16.7 ± 2.8	n.d.*	

***n.d.= not determined**

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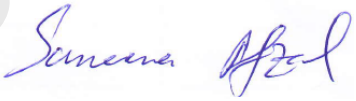

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