Research Article

Molecular Docking, Synthesis, and Tyrosinase Inhibition Activity of Acetophenone Amide: Potential Inhibitor of Melanogenesis

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Tyrosinase and its related proteins are responsible for pigmentation disorders, and inhibiting tyrosinase is an established strategy to treat hyperpigmentation. The carbonyl scaffolds can be effective inhibitors of tyrosinase activity, and the fact that both benzoic and cinnamic acids are safe natural substances with such a scaffolded structure, it was speculated that hydroxyl-substituted benzoic and cinnamic acid derivatives may exhibit potent tyrosinase inhibitory activity. These moieties were incorporated into new chemotypes that displayed in vitro inhibitory effect against mushroom tyrosinase with a view to explore antimelanogenic ingredients. The most active compound, 2-((3-acetylphenyl)amino)-2-oxoethyl(E)-3-(2,4-dihydroxyphenyl)acrylate (5c), inhibited mushroom tyrosinase with an IC50 of 0.0020 ± 0.0002 μM, while 2-((3-acetylphenyl)amino)-2-oxoethyl 2,4-dihydroxybenzoate (3c) had an IC50 of 27.35 ± 3.6 μM in comparison to the positive control arbutin and kojic acid with a tyrosinase inhibitory activity of IC50 of 191.17 ± 5.5 μM and IC50 of 16.69 ± 2.8 μM, respectively. Analysis of enzyme kinetics revealed that 5c is a competitive and reversible inhibitor with dissociation constant (Ki) value 0.0072 μM. In silico docking studies with mushroom tyrosinase (PDB ID 2Y9X) predicted possible binding modes in the enzymatic pocket for these compounds. The orthohydroxyl of the cinnamic acid moiety of 5c is predicted to form hydrogen bond with the active site side chain carbonyl of Asn 260 (2.16 Å) closer to the catalytic site Cu ions. The acetyl carbonyl is picking up another hydrogen bond with Asn 81 (1.90 Å). The inhibitor 5c passed the panassay interference (PAINS) alerts. This study presents the potential of hydroxyl-substituted benzoic and cinnamic acids and could be beneficial for various cosmetic formulations.
1. Introduction

A binuclear copper containing metalloenzyme tyrosinase has been detected in various species including fungi, bacteria, plants, and animals [1]. It is primarily involved in catalytic oxidation of its natural substrates L-tyrosine and L-DOPA (L-3,4-dihydroxyphenylalanine) to dopaquinone in a rate-limiting step during melanin biosynthesis which is associated with the reduction of oxygen (O2) into water [2, 3]. The dopaquinone synthesized in vivo in specialized organelles called melanosomes undergo further oxidation and several nonenzymatic polymeric reactions to form brown/black eumelanin and by reacting with cysteine and thiols to yellow/red pheomelanin [4–6]. Melanin is mainly responsible for skin, hair, and eye color and protects DNA degradation by ultraviolet (UV) radiation by acting as a photoprotectant in living organisms. The UV rays initiate melanogenesis for protecting skin cells from detrimental effects [7]. However, an abnormal production and accumulation of melanin in the dermal layer could cause several dermatological disorders including melanoma, freckle, lentigo, and Riehl melanosis [8, 9]. To date, numerous approaches including the tyrosinase inhibition, melanocyte to keratinocyte melanin transfer suppression, and cell signaling inter-

2. Materials and Methods

2.1. General. Dry dichloromethane (DCM) was dried following the standard method. The IR-spectra (cm⁻¹) were taken with a FT-IR spectrophotometer (Perkin Elmer, USA) using attenuated total reflection sampling technique (ATR). NMR spectra were taken using a Bruker spectrometer (1H, 400 MHz, 13C, 100 MHz, DMSO-d₆), and chemical shifts (δ) are reported in parts per million (ppm). The Flash elemental analyzer connected with thermal conductivity detector (Flash-TCD 2000 Series, USA) was used to perform elemental analysis (C, H) and reported uncorrected with ±0.3%. The melting points determined by Digimelt MPA 160 (USA) are reported uncorrected. Silica gel thin layer column chromatography-TLC was used to check the purity of title derivatives (petroleum spirit:ethyl acetate, 2:1). All other reagents such as the enzyme mushroom tyrosinase (Mtyr, EC 1.14.18.1), L-DOPA, and 1-(3-acetylphenyl)ethan-1-one were analytical grade (Sigma Aldrich, USA) and are used without further purification.

2.2. Chemistry

2.2.1. General Procedure for the Synthesis of N-(3-Acetylphenyl)Benzoates 3(a–e) and Cinnamates 5(a–e). The intermediate N-(3-acetylphenyl)-2-chloroacetamide (2) synthesized by following Sidhu et al., with little modifications [32], was condensed with various hydroxyl-substituted benzoic acids 2(a–e) and cinnamic acids 4(a–e) (0.01 mol) with equimolar triethylamine and potassium iodide (KI) in dimethylformamide (DMF) (25 mL) under nitrogen at 25°C and stirred overnight. The reaction mixture was then concentrated using rotary evaporator and extracted using ethyl acetate (3 × 25 mL). Finally, the combined ethyl acetate layer was treated with HCl (5%) and brine followed by drying over magnesium sulphate MgSO₄, filtered and concentrated by rotary evaporator to get the crude title products 3(a–e) and 5(a–e) (Schemes 1 and 2) and further purified by normal phase column chromatography (petroleum spirit:ethyl acetate, 3:1).

2.2.2. The Spectroscopic Characterization of Synthesized Compounds 3(a–e) and 5(a–e). The 1H-NMR of final compounds 3(a–e) and 5(a–e) showed an overlap of peaks in the alkyl region for homologues of this series. The singlet of terminal ketonic methyl proton of peripheral phenyl ring occupies the most upfield region of the spectrum encompassing the range of 2.54-2.56 ppm. In 13C NMR, the carbon of this terminal ketonic methyl group resonates between chemical shifts 26.73 and 26.80 ppm. The most shielded protons in the peripheral chain were the methylene (–CH₂) proton which are directly linked to ester groups. This methylene group has a chemical shift value of 4.67-4.94 ppm while the carbon shift of this group in 13C NMR was found between 61.92 and 63.13 ppm. The acetyl phenyl amide lies in the range 2901, 2865, 1725 (CO ester), 1632 (CO amide), 1589 (C=C, C=C) and 1403, 1387, 1359, 1278, 1246, 1221, 1152, 1138, 1099, 1071, 1021, 1002, 979, 906, 873, 864, 739, 687, 685, 677, 626, 515, 473, 416, 365, 342, 324, 290, 266, 250 ppm. The carbon shift of this group in 13C NMR was found between 165.37-172.06 ppm.

(1) (3a) 2-(3-Acetylphenylamino)-2-Oxethyl Hydroxybenzoate Solid; Mp: 138-140°C, Rf= 0.57, FT-IR (cm⁻¹): 3421, 3245, 2901, 2865, 1725 (CO ester), 1632 (CO amide), 1589 (C=C, C=C, C=C) and 1403, 1387, 1359, 1278, 1246, 1221, 1152, 1138, 1099, 1071, 1021, 1002, 979, 906, 873, 864, 739, 687, 685, 677, 626, 515, 473, 416, 365, 342, 324, 290, 266, 250 ppm. The carbon shift of this group in 13C NMR was found between 165.37-172.06 ppm.
(2) (3b) 2-(3-Acetylphenylamino)-2-Oxoethyl 4-Hydroxybenzoate. Solid; Mp: 146-148°C; Rf: 0.56, FT-IR (cm⁻¹): 3456, 3245, 2934, 2867, 1723 (CO ester), 1649 (CO amide), 1594 (C=C aromatic); 1H-NMR; δ =10.51 (1H, s, NH), 10.42 (1H, s, OH), 8.18 (s, 1H), 7.89 (d, J=8.8 Hz, 2H), 7.83 (d, J=6.8 Hz, 1H), 7.68 (d, J=8.1 Hz, 1H), 7.48 (t, J=6.1 Hz, 1H), 6.89 (d, J=8.7 Hz, 2H), 4.88 (2H, s, CH₂), 2.55 (3H, s, CH₃); 13C-NMR; δ =197.57 (CO, ketone), 168.28 (CO, amide), 165.37 (CO, ester), 150.79, 145.14, 138.93, 137.39, 132.13, 129.37, 126.74, 118.44, 118.15, 108.48, 103.86, 102.58, 62.85 (CH₂), 26.74 (CH₃) (Figure S3); Molecular formula: C₇H₁₅NO₆; Elemental Analysis: Calculated: (C, H) 62.00, 4.59; Found: (C, H) 62.02, 4.61.

(3) (3c) 2-(3-Acetylphenylamino)-2-Oxoethyl 3,4-Dihydroxybenzoate. Solid; Mp: 188-190°C; Rf: 0.49, FT-IR (cm⁻¹): 3423, 3234, 2923, 2867, 1727 (CO ester), 1637 (CO amide), 1596 (C=C aromatic); 1H-NMR; δ =10.41 (1H, s, NH), 8.17 (d, J=8.7 Hz, 1H), 7.44 (s, 2H), 7.40 (dd, J=2.0, 8.1 Hz, 1H), 7.44 (s, 2H), 6.85 (d, J=8.1 Hz, 1H), 4.86 (2H, s, CH₂), 4.28 (2H, s, OH), 2.56 (3H, s, CH₃); 13C-NMR; δ =197.60 (CO, ketone), 166.10 (CO, amide), 165.37 (CO, ester), 159.40, 158.14, 138.93, 137.39, 129.37, 123.85, 122.26, 119.98, 118.53, 118.15

Scheme 1: Synthetic pathway for title derivatives 3(a–e); reaction conditions and reagents; (i) dichloromethane (CH₂Cl₂)/triethylamine (C₂H₅)₃N, 0–5°C, 5 hours stirring (ii) dimethylformamide (DMF)/(C₂H₅)₂N/KI, stirring for 24 hours at room temperature.

Scheme 2: Synthetic pathway for title compounds 5(a–e); reaction conditions and reagents; (ii) dimethylformamide (DMF)/(C₂H₅)₂N/KI, stirring for 24 hours at room temperature.
113.39, 62.77 (CH\textsubscript{2}), 26.375 (CH\textsubscript{3}) (Figure S4); Molecular formula: C\textsubscript{19}H\textsubscript{19}NO\textsubscript{5}; Elemental Analysis: Calculated: (C, H) 66.85, 5.61; Found: (C, H) 66.83, 5.59.

2.3. Mushroom Tyrosinase Inhibitory Activity. In vitro antityrosinase potential was determined following the method previously described with little modifications [33, 34]. Briefly, 140 \mu L of sodium phosphate buffer, 20 \mu L (30 U/mL) of the enzyme mushroom tyrosinase, and 20 \mu L of the tested compound were added in a well of a 96-well microplate. After a preincubation of 10 minutes at room temperature of 20 °C (0.85 mM), the substrate L-DOPA was added followed by an incubation of 20 minutes at the same temperature. Subsequently, the absorbance of the intermediate dopachrome was recorded by using an Opti Max tunable microplate reader (Sunnyvale, USA) at 475 nm. The standard inhibitors were kojic acid and arbutin. The negative inhibitor was a phosphate buffer. All the compounds were dissolved in DMSO, and blank tests were performed with DMSO. IC\textsubscript{50} values were calculated by Origin (8.6, 64-bit).

2.4. Mushroom Tyrosinase Inhibitory Kinetics. Based upon the antityrosinase assay, the most potent derivative (5c) was chosen for kinetic inhibition studies using enzyme and L-DOPA as a substrate [35–37]. Various concentrations of the inhibitor 5c were among 0.00 to 0.013 \mu M during all kinetic assays.
inhibitory action and the kind of inhibition were determined by the Lineweaver-Burk and Dixon plots. $1/V$ (inverse of velocities) versus $1/[S]$ (inverse of substrate concentration) mM$^{-1}$ were plotted to determine inhibition constant $K_i$.

2.5. Computational Studies

2.5.1. Ligand Preparation. ChemDraw Professional 15.2 was used to sketch all the ligands and LigPrep (Schrödinger) to prepare in their neutral form. The conformation of the prepared ligands was optimized in the OPLS-3 force field for further docking analysis.

2.5.2. Retrieval of Mushroom Tyrosinase in Maestro. For this purpose, the crystal structure of target protein (PDB ID 2Y9X) was retrieved from RCSB and prepared in “Protein Preparation Wizard” workflow in Maestro Schrödinger for adding hydrogens, adjusting protonation and bond orders appropriate for pH 7. The water molecules beyond 5 Å from het groups were removed and minimized the prepared protein structure for pH 7. The water molecules beyond 5 Å from het groups were removed and minimized the prepared protein structure for further grid generation and docking analysis.

2.5.3. Receptor Grid Generation and Docking. For grid generation, the catalytic pocket is selected from its cocrystallized ligand and literature [38]. The grid was generated by specifying the cocrystallized ligand tropolone of the active site of the target protein. The receptor grid box was defined as 20 Å box. After grid preparation, Glide_dock_XP precision docking experiment was performed with default docking setup parameters reporting the 15 top-ranked poses per ligand [39]. The predicted binding scores (binding energies) and proper orientation of ligands within catalytic region of tyrosinase was also performed. Finally, the most favorable binding mode of active compounds within the binding pocket was investigated in terms of docking score and 3D graphical images of the binding pose of the best docked score was also done with Maestro (Schrödinger).

3. Results and Discussions

3.1. In Vitro Mushroom Tyrosinase Inhibition Assay. Benzoic and cinnamic acid analogues 3(a–e) and 5(a–e) were designed and synthesized to evaluate in vitro tyrosinase inhibition activity. Table 1 demonstrates that title amide and proper orientation of ligands within catalytic region of tyrosinase was also performed. Finally, the most favorable binding mode of active compounds within the binding pocket was investigated in terms of docking score and 3D graphical images of the binding pose of the best docked score was also done with Maestro (Schrödinger).

### Table 1: The yield, substitution pattern, mushroom tyrosinase inhibition and docking scores of analogues 3(a–e) and 5(a–e).

| Compounds | Yield (%) | Substitution pattern | Mushroom tyrosinase inhibition | Docking score (kcal/mol), PDB ID 2Y9X |
|-----------|-----------|---------------------|--------------------------------|----------------------------------------|
| 3a        | 81        | 3-OH                | $322.68 \pm 16.9$ M)          | -3.262                                 |
| 3b        | 78        | 4-OH                | $287.53 \pm 32.2$ M)          | -4.295                                 |
| 3c        | 74        | 2,4-di-OH           | $27.35 \pm 3.6$ M)            | -5.564                                 |
| 3d        | 69        | 3,4-di-OH           | $127.65 \pm 8.5$ M)           | -4.569                                 |
| 3e        | 76        | 3,5-di-OH           | $255.31 \pm 14.2$ M)          | -3.984                                 |
| 5a        | 75        | -H                  | $46.43 \pm 5.8$ M)            | -6.173                                 |
| 5b        | 73        | 4-OH                | $309.20 \pm 36.8$ M)          | -4.264                                 |
| 5c        | 71        | 2,4-di-OH           | $0.0020 \pm 0.0002$ M)        | -6.568                                 |
| 5d        | 78        | 4-cl                | $61.62 \pm 11.4$ M)           | -5.299                                 |
| 5e        | 72        | 4-OH                | N.d.*                          | -4.628                                 |
| Arbutin   |           |                     | $191.17 \pm 5.5$ M)           | -4.759                                 |
| Kojic acid|           |                     | 16.69 ± 2.8                   | -3.792                                 |

3a: 2-(3-acetylphenoxy)-2-oxoethyl-3-hydroxybenzoate; 3b: 2-(3-acetylphenoxy)-2-oxoethyl-4-hydroxybenzoate; 3c: 2-(3-acetylphenoxy)-2-oxoethyl-2,4-dihydroxybenzoate; 3d: 2-(3-acetylphenoxy)-2-oxoethyl-3,4-dihydroxybenzoate; 3e: 2-(3-acetylphenoxy)-2-oxoethyl-3,5-dihydroxybenzoate; 5a: 2-(3-acetylphenoxy)-2-oxoethylcinnamate; 5b: 2-(3-acetylphenoxy)-2-oxoethyl(4-hydroxyphenyl) acrylate; 5c: 2-(3-acetylphenoxy)-2-oxoethyl(E)-3-(4-chlorophenyl) acrylate; 5d: 2-(3-acetylphenoxy)-2-oxoethyl(E)-3-(4-chlorophenyl) acrylate; 5e: 2-(3-acetylphenoxy)-2-oxoethyl(E)-3-(4-hydroxyphenyl) propanoate; SEM: the standard error mean; N.d.*: not determined.
respectively, whereas the inhibitor 5d bears a chloro substituent at the para position. The hydroxyl substitution at the second and fourth positions of the cinnamic acid phenyl ring in 5c resulted in a considerable rise in the enzyme inhibitory activity (Table 1). The phenolic hydroxyls play a crucial role in tyrosinase inhibition as the natural substrates of tyrosinase, L-tyrosine, and L-DOPA also bear the phenolic hydroxyls [40]. A naturally occurring cinnamic acid has been reported with extensive physiological activities including tyrosinase inhibitory action [41]. The compounds 5a (IC$_{50}$ 46.43 μM), 5b (IC$_{50}$ 309.20 μM), and 5d (IC$_{50}$ 61.62 μM) displayed lower inhibitory activity than 5c (IC$_{50}$ 0.0020 μM) compared to reference inhibitor kojic acid (IC$_{50}$ 16.69 μM) and arbutin (IC$_{50}$ 191.17 μM). It is reported here that the ortho- and parahydroxyls of the phenyl ring in 5c appear to show an increased tyrosinase inhibitory activity (Figure 1).

3.2. Enzyme Inhibitory Kinetics. The enzyme inhibitory interaction mechanism of 3(a–e) and 5(a–e) with the bind-
The site of mushroom tyrosinase was determined using Michaelis-Menten kinetic studies. The inhibitors exhibit a dose-dependent inhibition of the enzyme tyrosinase. A striking reduction in the reaction rate in the presence of inhibitors refers to a decrease in the final absorbance in comparison to control without inhibitors. The inhibitory index of all these tested compounds varied depending upon the position of various substituents and classes of compounds [27]. Inhibition kinetics was analyzed by the Lineweaver-Burk and Dixon plots. The kinetics analyses of 1/V max vs. 1/[S] at different doses of 5c reveal that the Michaelis-Menten constant (Km) changes while that of 1/V max remained the same representing the competitive nature of the most potent inhibitor (5c) (Figure 2(a)). The dissociation constant Ki for 5c was 0.0072 μM calculated by Dixon plots as shown in Figure 2(b) [27, 42].

3.2.1. Inhibitory Action of Compound 2-(3-Acetylphenylamino)-2-Oxoethyl (E)-3-(2,4-Dihydroxyphenyl) Acrylate (5c) Using L-DOPA as Substrate. The plot of the remaining enzyme activity at different concentrations (0.00, 1.25, 2.5, and 5 μg/mL) of enzyme vs. various doses (0.00, 0.0004, 0.0008, 0.0016, 0.0032, 0.0065 μM) of the inhibitor 5c for L-DOPA catalysis showed a group of straight lines with varying slopes and intersecting on the same point on the y-axis preferring the reversible effect of 5c on tyrosinase (Figure 3). The results suggested that 5c effectively inhibits the enzyme tyrosinase by binding to its active site reversibly [35].

3.3. In Silico Studies

3.3.1. Binding Pocket Analysis of Analogue 3(a-e) and 5(a-e) against Mushroom Tyrosinase. A computational molecular docking study was also conducted to examine the binding conformations of all the synthesized compounds within the catalytic pocket of enzyme tyrosinase. The docked ligand-protein complexes were investigated based on docking score (kcal/mol) and the hydrophobic/philic bonding interaction pattern. The docking scores had little fluctuations, and the comparison depicted no significant energy difference among all docked molecules due to similar basic skeleton of the ligands. Therefore, majority of the ligands showed efficient docking energy values. From docking results and in vitro enzyme inhibitory assay, the most active compound 5c (-6.568 kcal/mol) was visualized to determine its interactions in the catalytic site of the protein tyrosinase (Figure 4). Two
Table 2: Pharmacokinetic assessment of synthesized drugs 3(a–e) and 5(a–e).

| Compound | M.Wt ≤ 500 | HBA ≤ 10 | HBD ≤ 5 | LogP ≤ 5 | LogS (mol/L) ≤ –4 | PSA ≤ 140 (Å²) | Vol (Å³) | Drug likeness score > 0 | ROS |
|----------|------------|----------|---------|----------|-------------------|----------------|----------|------------------------|-----|
| 3a       | 313.10     | 5        | 2       | 2.54     | -2.65             | 75.69          | 301.07   | 0.16                   | Yes |
| 3b       | 313.10     | 5        | 2       | 2.51     | -2.78             | 75.69          | 300.99   | 0.49                   | Yes |
| 3c       | 329.09     | 6        | 3       | 2.76     | -3.23             | 92.24          | 312.25   | 0.74                   | Yes |
| 3d       | 329.09     | 6        | 3       | 1.96     | -2.40             | 91.17          | 313.72   | 0.60                   | Yes |
| 3e       | 329.09     | 6        | 3       | 2.10     | -2.62             | 93.31          | 311.77   | -0.08                  | Yes |
| 5a       | 323.12     | 4        | 1       | 3.52     | -3.67             | 57.48          | 334.19   | -0.60                  | Yes |
| 5b       | 339.11     | 5        | 2       | 2.96     | -3.33             | 75.09          | 344.74   | -0.05                  | Yes |
| 5c       | 355.11     | 6        | 3       | 2.72     | -3.11             | 91.64          | 355.34   | -0.08                  | Yes |
| 5d       | 357.08     | 4        | 1       | 4.12     | -4.73             | 57.48          | 351.39   | -0.04                  | Yes |
| 5e       | 341.13     | 5        | 2       | 2.82     | -3.30             | 75.09          | 336.60   | -0.01                  | Yes |
| Kojic acid | 142.03  | 4        | 2       | 1.07     | -0.08             | 56.19          | 148.32   | -1.04                  | Yes |
| Arbutin  | 272.09     | 7        | 5       | -1.14    | -0.96             | 97.71          | 228.69   | -1.04                  | Yes |

M.Wt: molecular weight; HBA: number of hydrogen bond acceptor; HBD: number of hydrogen bond donor; LogP: partition coefficient; LogS: lipophilicity of water; PSA: total polar surface area; RO5: Lipinski’s rule of 5.

strong hydrogen bond contacts were observed. The ortho-hydroxyl phenolic moiety of 5c is picking up a hydrogen bond (2.16 Å) interaction with the side chain carbonyl of Asn 260, and this phenolic ring is further stabilized by π-π stacking with side chain His 259 and His 263. The keto carbonyl oxygen from the tail moiety of this compound is interacting with side chain His 259 and His 263. The keto carbonyl oxygen (2,4-dihydroxy phenyl)acrylate (5c) exhibited the most promising inhibitory activity against tyrosinase (IC50 16.69 μM) better than the standard kojic acid (IC50 16.69 μM) and arbutin (IC50 191.17 μM). Moreover, in the cinnamate amide 5(a–e) scaffold, the presence of –OH groups is of great interest for antityrosinase activity. In addition, from benzoic acid analogues, 3c (2-((3-Acetylphenylamino)-2-oxoethyl 2,4-dihydroxy benzoate) also displayed considerable activity against tyrosinase (IC50 27.35 μM) and has potential to be explored further for antityrosinase and cosmetic drug discovery and its clinical exploitation.

Data Availability

Data could be provided upon request from the shared corresponding author Dr. Zaman Ashraf, mzchem@yahoo.com

Conflicts of Interest

The authors declare that they have no known conflict of interest to influence the work reported in this research.

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Supplementary Materials

Figures S1-S10 are available online as supplementary materials. (Supplementary Materials)

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