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Hydroxyphenyl thiosemicarbazones as inhibitors of mushroom tyrosinase and antibrowning agents.

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Hydroxyphenyl thiosemicarbazones as inhibitors of mushroom tyrosinase and antibrowning agents / Carcelli, M; Rogolino, D; Bartoli, J; Pala, N; Compari, C; Ronda, N; Bacciottini, F; Incerti, M; Fisicaro, E.. - In: FOOD CHEMISTRY. - ISSN 1873-7072. - 303:(2020), p. 125310. [10.1016/j.foodchem.2019.125310]

Availability: This version is available at: 11381/2862696 since: 2024-12-13T12:12:09Z

Publisher: Elsevier Ltd

Published DOI:10.1016/j.foodchem.2019.125310

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22 **Keywords**: tyrosinase inhibitor; thiosemicarbazone; antibrowning activity; food browning;

- 23 equilibrium constant; kojic acid
- 24

25 Abstract

26 Tyrosinase is a metalloenzyme involved in o-hydroxylation of monophenols and oxidation of odiphenols to o-quinones, with formation of brown or black pigments (melanines). Tyrosinase 27 inhibitors are of great interest in medicine and cosmetics (skin whitening compounds), but also in 28 food and beverage industry (antibrowning agents). Here we report on the activity as mushroom 29 tyrosinase inhibitors of a series of hydroxyphenyl thiosemicarbazones (1-5): one of them revealed an 30 31 inhibitory activity stronger than kojic acid, used as reference. Enzymatic inhibition activity was confirmed by colorimetric measurements on small wheels of Fuji apples treated with the 32 hydroxyphenyl thiosemicarbazones. The mechanism of action of compounds 1-5 was investigated by 33 34 molecular modelling and by studying in solution their speciation with Cu(II) ions, the ions in the 35 active site of the enzyme. Finally, compounds 1-5 were tested on human fibroblasts: they are not cytotoxic and they do not activate cells in a pro-inflammatory way. 36

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39 **1. Introduction**

Tyrosinase is a metalloenzyme that controls the production of melanin, a broad term for a group of
natural pigments found in humans, animals and plants. Melanin is responsible for the color of human
skin, but it is, for example, also involved in the enzymatic browning of fresh-cut fruits and vegetables.
As a consequence, tyrosinase inhibitors could be of interest as skin whitening compounds, but also

as antibrowning agents for food and beverage industry (Zolghadri et al., 2019). In the last decades 44 45 the change in lifestyle and the increase in health consciousness and in purchase power of developed countries lead to a fast growth of the ready-to-eat fruit and vegetable market. Notwithstanding the 46 minimal processing that fresh fruits and vegetables undergo, these products present a reduced shelf 47 48 life, due, among others, to enzymatic browning (Croguennec, T., 2016). In order to help to increase the shelf life and preserve natural and appealing appearance and organoleptic fresh-like 49 50 characteristics, the fruit processing industry requires the development of new, efficient and safe tyrosinase inhibitors, to be included in the packaging materials or in edible coatings. Consumers are 51 influenced in their choices by the color of fruits and vegetables, that are exposed to enzymatic 52 53 browning because of brushing, peeling, and crushing operations (Loizzo, Tundis & Menichini, 2012).

A large number of tyrosinase inhibitors have already been reported in the literature, from both 54 synthetic and natural sources (Zolghadri et al., 2019; Pillaiyar, Namasivayam, Manickam & Jung, 55 2018; Chan, Huang, Lee & Lin, 2014; Ullah, Son, Young Yun, Hyun Kim, Chun & Moon, 2016; 56 57 Kostopoulou & Detsi, 2018), but most of them are not suitable for practical applications, because of 58 safety reasons, their scarce activity or their off-flavors. Actually, only few antibrowning agents are 59 commonly used by food industry, such as ascorbic and citric acids. Then, it has been recently noted that only a few of available studies are well sounded from chemical and mechanistic point of view 60 61 (Tang, Cui, Li, Huang & Li, 2018; Xu, Liu, Zhu, Yu & Cao, 2017). Thiosemicarbazones (TSCs) have a lot of different biological activities, and they have been also proposed as inhibitors of mushroom 62 tyrosinase (Yi et al. 2011; Xu, Liu, Zhu, Yu & Cao, 2017; Chen et al., 2012; Hałdys et al., 2018). 63 Metalloenzymes could be inhibited by complexation of the metal ion/ions in their active site; in other 64 65 words, the inhibition of the metalloenzyme depends on the possibility for the inhibitor to interact with 66 the entire enzymatic cleft, and, in particular, to coordinate the metal ion/ions in the active site (Chen, 67 Adamek, Dick, Credille, Morrison & Cohen, 2019). It is well known that the thiosemicarbazone group has a good affinity for the copper ions, and tyrosinase, that belongs to the Type-3 copper protein 68

family, has two copper ions in the active site. We have decided to study the activity of a series of 69 70 TSCs, introducing the hydroxyphenyl group on their skeleton, since phenolic compounds are known to be good tyrosinase inhibitors (Pillaiyar, Namasivayam, Manickam & Jung, 2018). Moreover, when 71 the OH is in *ortho* on the phenyl ring, it can contribute to the chelation of the metal ion, or, if it is in 72 a different position, it can establish interactions with the aminoacidic residues of the enzymatic cleft. 73 Finally, the terminal thiosemicarbazone NH₂ was modified to slightly improve water solubility, 74 75 obtaining the pool of compounds 1-5 (Figure 1), that were tested as mushroom tyrosinase inhibitors. Docking and solution studies were performed in order to have information about structural features 76 impairing enzymatic inhibition activity. The cytotoxic profile was also evaluated. We studied the 77 78 potential non-specific toxicity toward human tyrosinase-negative (fibroblasts) of compounds 1-5 as 79 well as their ability to induce a pro-inflammatory activation in the same cells.

80

81

82 **2.** Materials and methods

83 2.1. Materials

All reagents were of analytical grade and were used as received. The purity of the synthesized
compounds was verified ≥95 % by elemental analysis. Technical data about NMR, ATR-IR,
electrospray mass spectral analyses (ESI-MS), and ICP analyses were already reported (Rogolino et
al., 2015).

88

89 2.2. Synthesis

90 The TSCs 1-5 were prepared following reported literature procedures (Rogolino et al., 2015). Briefly,
91 thiosemicarbazide in hot absolute ethanol was mixed with an equimolar amount of aldehyde, in the
92 same solvent. The solution was refluxed for 6 h, cooled at room temperature, and then concentrated

in vacuum. The precipitate was filtered off, washed with cold ethanol and dried in vacuum.
Characterizations (¹H-NMR, IR, MS, elemental analysis) of the TSCs 1-5 are reported in the
Supplementary Material.

96

97 2.3 Tyrosinase activity

98 The mushroom tyrosinase used for the bioassay was purchased from Sigma-Aldrich. The diphenolase 99 activity of tyrosinase was checked according to the method of Masamoto (Masamoto, Ando, Murata, Shimoishi, Tada & Takahata, 2003) with minor modifications, using L-DOPA (Sigma-Aldrich) as 100 101 substrate. Enzyme activity was determined at room temperature following the increase in absorbance at 475 nm ($\epsilon = 3700 \text{ M}^{-1} \text{ cm}^{-1}$) determined by the formation of dopachrome by a Kontron Uvikon 860 102 spectrophotometer. Stock solutions of L-DOPA (15 mM), inhibitor (0.5 mM) and enzyme (2.5 U/µl) 103 104 were prepared in Na₂HPO₄-NaH₂PO₄ 25 mM buffer (pH 6.8) and then diluted to the appropriated test concentrations. The experiments were performed at a constant enzyme concentration (12.5 U/mL), 105 varying inhibitor and substrate concentrations. The substrate was added at the last moment to the 106 reaction mixture and immediately absorbance measurement started for measuring the initial reaction 107 rate from the slope of the linear increase of optical density. 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-108 109 4-one (kojic acid, Sigma-Aldrich) was used as positive control.

110

111 2.4 Colorimetric measurements

The colorimetric measurements were performed using Fuji apples, following the method of Wu *et al.*(Wu, Cheng, Li, Wang & Ye, 2008). Apples were washed and cut into small wheels by a mandolin
slicer. After that, they were dipped in 200 mL of 30 μM test solution, prepared in Na₂HPO₄-NaH₂PO₄
25 mM buffer (pH 6.8) for 3 min and then drained. This procedure was repeated for the compounds **1**, **2**, **3** and **5**. Control samples were dipped in Na₂HPO₄-NaH₂PO₄ 25 mM buffer alone. Samples were

then placed on absorbent paper and stored at room temperature. Changes of color were measured with a tristimulus reflectance colorimeter (TECHKON SpectroDens – Spectro-Densitometer), calibrated using a standard white reflector plate. The readings were made for each replicate by changing the position of the apple small wheel to get uniform color measurements. The CIE $L^* a^* b^*$ color space was assumed, where L^* indicates lightness, a^* indicates chromaticity on a green (-) to red (+) axis, and b^* chromaticity on a blue (-) to yellow (+) axis. Measurements were made immediately after dipping and at time intervals thereafter. Total color difference (ΔE^*) calculated as follows:

124
$$\Delta \mathbf{E}^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

125 was also used to evaluate the antibrowning potential of different treatments.

126

127 2.5 Potentiometry

The Cu(II) stock solution was prepared from CuCl₂·2H₂O (Merck). Its concentration was determined 128 by titrating the solution with EDTA in presence of concentrate ammonia; Fast Sulfon Black is used 129 as indicator. Equilibrium constants at 25 ± 0.1 °C for protonation and complexation reactions were 130 determined as detailed in Rogolino et al., 2015. Temperature was controlled to ±0.1 °C by a ISCO 131 GTR 2000 IIx bath. Appropriate amounts of ligand solution, prepared by weight, were titrated with 132 standard KOH or HCl (when starting from the alkaline solution pH \approx 11), with and without metal ions, 133 under magnetic stirring. The protonation constants were obtained by titrating 20 or 50 ml of samples 134 of each ligand (0.05 mmol). The speciation was defined by performing the titrations at different 135 ligand/metal ratios (1 up to 4). At least two measurements (about 60 experimental points each) were 136 performed for each system. The electrodic chain was calibrated, again, as detailed in Rogolino et al.. 137 Speciation and the logarithm of the stability constants (log β_{pqr}) are obtained by using the program 138 HYPERQUAD (Gans, Sabatini, & Vacca, 1996). In $\beta_{pqr} = [M_p L_q H_r]/[M]^p [L]^q [H]^r$, the equilibrium 139

140 constant for $pM + qL + rH = M_pL_qH_r$, M is the metal, L the fully deprotonated ligand, H the proton, 141 and charges are not reported for simplicity.

142

143 *2.6 Cellular assays*

Human skin fibroblasts, obtained with informed consent from a healthy male subject, were cultured 144 medium, i.e. DMEM (Lonza), 145 in standard culture supplemented with 100 U/mL penicillin/streptomycin (Life Technologies) and 10% fetal calf serum (Sigma Aldrich), in 96-well 146 plates at 5% CO₂ and 37°C. When confluent, cells were incubated in triplicate with sterile filtered 147 standard culture medium with or without 1-5 100 µM for 24 hours. Cell viability was then evaluated 148 149 through the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1993). Briefly, cells were incubated with 1 mg/ml MTT (Sigma Aldrich) for two hours. Cells were 150 then washed with phosphate buffer saline at 37 °C and treated with dimethyl sulfoxide (Sigma 151 Aldrich) in the dark. Cell lysates absorbance at 570 nm was measured with a Spark 10M TECAN 152 microplate reader. 153

154 Cell pro-inflammatory activation was evaluated measuring the concentration of interleukin-6 (IL-6) 155 in cell supernatants after 24 hours incubation with standard culture medium with or without 100 μ M 156 solutions of 1-5. IL-6 was measured using a commercially available ELISA kit (Thermo Scientific).

157

158 2.7 Molecular modelling

159 2.7.1 Ligands preparation

Molecular Operating Environment software package platform (MOE) was used for the current computational study (Molecular Operating Environment, MOE 2009). A single database file containing the ligands for molecular docking studies was prepared using MOE builder mask. The geometries of the selected ligands were optimized by an energy minimization pass until a 164 convergence gradient criterion of 0.0001 kcal mol⁻¹ Å⁻¹ was reached, using the MMFF94x force field.
165 Solvent effect was calculated using the Generalized Born Solvation Model.

166 2.7.2 Protein preparation

167 The X-ray coordinates of a tyrosinase from *Agaricus bisporus* in complex with inhibitor tropolone 168 (PDB code: 2Y9X) (Ismaya et al., 2011) were retrieved from RCSB Protein Data Bank 169 (<u>http://www.rcsb.org/</u>). Before docking protein termini were capped and solvent water molecules were 170 removed; then the system was 3D protonated and partial atomic charges were assigned according 171 OPLS_AA force field.

172 2.7.3 Docking procedures

The active site pocket was defined in order to contain the residues within 15 Å from the cocrystallized tropolone molecule. The different docking methods implemented in MOE platform were tested by re-docking using different combinations of placement, rescoring and refinement features, and the following conditions were chosen: Alpha Triangle as placement method, London dG as rescoring 1 function, Forcefield as refinement, and Affinity dG as second rescoring procedure. The same docking procedure was applied to the ligands, and the highest docking pose of each ligand was retained.

180

181 **3. Results and discussion**

The TSCs 1-5 were easily prepared in good yields by condensation of the hydroxyphenyl aldehyde and thiosemicarbazide (5) or 4-ethyl-3-thiosemicarbazide (1-4) (Figure 1) (Rogolino et al., 2015). The ethyl derivatives are slightly more soluble in water, where tyrosinase inhibition tests are carried out. 1-5 differ for number (two or three) and positions of the OH groups on the phenyl moiety bound to the TSC scaffold, offering different possibilities for the interactions with the enzyme. 188 *3.1 Effect on tyrosinase activity*

189 Measurements were carried out as a function of the substrate concentration at different inhibitor 190 concentrations, as described in paragraph 2.3. Kojic acid, one of the most studied tyrosinase inhibitor 191 (Bentley, 2006), was assumed as reference.

192 Compounds 3 and 4, with two hydroxyl groups in different positions of the phenyl ring (Figure 1), do not exhibit any inhibiting activity against mushroom tyrosinase. The inhibitory effect of 1, 2 and 193 5 is concentration-dependent, the enzyme activity decreasing with the increasing of the concentration 194 195 of the tested compounds, without being completely suppressed. In Figure 2 the Michaelis-Menten plots for the reaction without inhibitor and in presence of different concentrations of compounds 1 196 and 5 are shown (2 behaves in a very similar way as 1 and it is omitted for clarity). The type of 197 inhibition on the diphenolase activity was defined by the Linweaver-Burk plot (Copeland, 2000). The 198 active compounds show a noncompetitive (mixed type) inhibition, that is they bind to the enzyme not 199 necessarily by competing with the substrate. Since the affinity (K_i) of the inhibitor for the free enzyme 200 E is different from the affinity of the inhibitor (αK_i) for the complex enzyme-substrate ES (this means 201 that α is finite but $\neq 1$), it can be defined mixed inhibition (**Table 1**). The Lineweaver-Burk plot for 202 5, the most active compound, is reported as an example (Figure 2); the same plots for 1 and 2 can be 203 found in SI (Figure S1 and Figure S2). 204

Considering the reciprocal form of the most general velocity equation for noncompetitive inhibition(Copeland, 2000):

207
$$\frac{1}{\nu} = \left(\left(1 + \frac{[I]}{Ki}\right) \left(\frac{Km}{Vmax}\right) * \frac{1}{[S]} + \left(1 + \frac{[I]}{\alpha Ki}\right) * \frac{1}{Vmax} \right)$$

208 ([I] and [S] are the concentrations of the inhibitor and the substrate, respectively), the inhibition 209 constant K_i is obtained from the dependence of the slope of the Lineweaver-Burk plot from the 210 inhibitor concentration, and the value αK_i from the dependence of the intercept of the same plot from

the inhibitor concentration. Both plots are linear (**Figures 2c** and **2d**) and the above mentioned quantities are obtained from the *ratio* intercept/slope of the appropriate straight line. K_i is related to the inhibitor activity, being the greater the smaller is the value of K_i .

Among the studied TSCs, only **5** possesses an inhibitory activity of practical interest, stronger than those of the kojic acid assumed as reference, as revealed by the values of K_i and IC₅₀ (**Table 1**). IC₅₀ is the concentration of the inhibitor able to halve the maximum reaction rate in the adopted experimental conditions; the value reported for kojic acid agrees with the literature data, if normalized for the tyrosinase concentration (Qamar et al. 2019; Sari, Barut, Özel, Kuruuzum-Uz & Şohretoğlu, 2019; Şöhretoğlua et al., 2018; Larik et al. 2017).

The position of the substituents on the phenyl group plays a negligible role on the inhibitor activity (**Table 1**), whereas a little enhancement is obtained for the compounds **1** and **2** with three hydroxyls instead of two. Note that **4** and **5** differ only for the ethyl group bound to the terminal nitrogen in **4**: evidently, the removal of this group is essential for changing an inactive compound (**4**) in a strong tyrosinase inhibitor (**5**).

225

226 *3.2 Antibrowning treatment of Fuji apple small wheels*

The effects of the dipping in 30 µM solutions of 1, 2, 3, and 5 are qualitatively shown in Figure 3, 227 228 where they are compared after 28 hours from dipping. Compound 3 was used to check if it has an 229 antibrowning effect on the apple wheels, even if it does not inhibit tyrosinase activity in the enzyme assay. The increase of browning follows, as expected, the reverse order of the inhibitory activity 230 231 against tyrosinase. The inactive compound 3, as expected, behaves in the same way as the control sample. On the contrary 5 seems to show the greatest antibrowning effect. It is much more difficult 232 to quantify the effect in terms of color differences by means of colorimetric measurements. Because 233 an increase of the parameter a* is related with an increase in redness, it has been suggested (Abbott 234

& Buta, 2002; Saper & Miller, 1998; Rojas-Grau, Sobrino-Lopez, Tapia & Martin-Belloso, 2006) 235 236 that this parameter could be the more discriminating one. However, our results show that all the colorimetric parameters are able to provide a similar information, and in particular a* (chromaticity 237 on a green (-) to red (+) axis) and b* (chromaticity on a blue (-) to yellow (+) axis); see in the 238 supporting information **Figure S3**. The samples are divided in two groups: those completely without 239 antibrowning activity (control sample and sample treated with 3) and those treated with a 240 241 thiosemicarbazone with antityrosinase activity (1, 2 and 5). Within the last group, the differences are not statistically significant, even if they are appreciable at naked eye. 242

243

244 *3.3 Potentiometry*

Tyrosinases, belonging to the type 3 copper-containing protein, contain two copper ions in the 245 conserved active site, coordinated by six histidine residues (Deri et al., 2016; Ramsden & Riley, 246 2014). It has been suggested (Sendovski, Kanteev, Ben-Yosef, Adir & Fishman, 2011; Noh, Kwak, 247 248 Seo, Seo, Kim & Lee, 2009; Lima et al., 2014; Bochot et al., 2014) that a possible mechanism of inhibition of tyrosinase activity is the chelation of the metal ions in the active site. This possibility 249 was deeply investigated for kojic acid, a well-known tyrosinase inhibitor (Noh, Kwak, Seo, Seo, Kim 250 251 & Lee, 2009; Lima et al., 2014; Bochot et al., 2014). Starting from these considerations, we have studied the solution equilibria of the TSCs 2, 4 and 5 with H⁺ and Cu(II) ions by potentiometry in 252 water at ionic strength 0.1 M KCl. In water these ligands are slightly soluble at acid pH, but they are 253 perfectly soluble in the millimolar range at alkaline pH; for this reason, the protonation constants 254 were obtained starting from pH \approx 11 and titrating by HCl. The TSCs 4 (pK₁=8.51, pK₂=10.53) and 5 255 $(pK_1=8.37, pK_2=10.42)$ result to be diprotic acids with similar values of pK_a (**Table 2**). These values, 256 particularly for pK₂, are lower than those reported for salicylaldehyde thiosemicarbazone, the 257 258 difference being due also to the different solvent used in the titrations (Enyedy et al. 2012). Compound 5, with three -OH groups on the phenyl ring, is a triprotic acid (Table 2: $pK_1=7.33$, $pK_2=7.95$, 259

pK₃=11.79). Even if the different proton-dissociation equilibria are difficult to separate, for **4** and **5**, the first dissociation constant can be reasonably attributed to one of the OH and the second one to the dissociation of the N-H group of the thiosemicarbazide moiety (Enyedy et al. 2012). For **2** both the constants pK_1 and pK_2 , very near as value, are probably attributable to two OH groups, and the third one to the N-H proton; the presence of two ionizable substituents reduces the acidity of the N-H group of about one order of magnitude.

The speciation and the values of the formation constants of the copper complexes were obtained by 266 titrating 2, 4 and 5 in presence of Cu(II) ions at different metal: ligand ratios (from 1 up to 6). Some 267 problems arise from the low solubility of the copper(II) complexes. In fact, the system 4:Cu(II) was 268 269 soluble in all the pH range studied, while a precipitate was formed from pH=3 to pH=7 for all the ligand:metal ratios investigated for 2. Unfortunately, the most active thiosemicarbazone 5 gives rise 270 271 to the formation of insoluble complexes in the whole examined pH range. It must be outlined that all 2, 4 and 5 possess in *orto* on the phenyl ring at least one OH group involved in the chelation of Cu(II) 272 ions. Table 2 displays the models for the speciation obtained by the Hyperquad software, showing 273 274 the best statistical parameters and the best fit between experimental and computed potentiometric 275 titration curves. Other models are also accepted by the software, in particular including only the 1:1 metal:ligand stoichiometry (Enyedy et al. 2012), but with worse statistical parameters and greater 276 standard deviations of the formation constants. 277

The complexes of **4** are already present at acidic pH with formation of the neutral CuL species, having a very high stability; at near pH = 8 CuL₂ begins to form. For a better comparison, we can consider also **2** as a diprotic acid, setting L'= LH. With this substitution it is easy to see that the sets of complexes formed in solution have the same stoichiometry for **2** and **4**, as expected, due to the presence of the same chelating moiety. For **2** the insoluble species seem to be CuL' (i.e. CuLH), corresponding to CuL for **4**. The distribution diagrams as a function of pH for L:Cu = 4:1 at a Cu(II) concentration equal to 1.25 mM clearly show that formation of CuL (CuLH, in the case of **2**) starts at low pH, confirming that TSCs are very efficient Cu(II) chelators (Figure S4) (Sendovski, Kanteev,
Ben-Yosef, Adir & Fishman, 2011). The chelation of the Cu(II) ion greatly increases the dissociation
of the hydrazine group in the complexes of 2 and 4, particularly for 2. The solubility of the Cu(II)
complexes follows a reverse order in comparison with the inhibitory activity, the complexes with 4
(without inhibitory activity) being soluble, and those with 5 (the most potent inhibitor) completely
insoluble. Copper complexes of kojic acid are also completely insoluble in the full range of pH, not
allowing to be studied in solution (Bhatia, Kociok-Kohn & Molloy, 2015).

In the active site of the enzyme, the inhibitor could coordinate both the copper(II) ions at the same time. To gain experimental evidence for the possible formation of bimetallic species in solution, we have performed the titrations even in great excess of Cu(II) in respect to the ligand, but the software always rejected the polinuclear species.

296

297 *3.4 Molecular modelling*

To predict the putative binding mode of the compounds **1- 5** with the tyrosinase catalytic site at molecular level, a series of computational docking studies was performed. First, optimized conformations of the compounds were obtained using the MOE (Molecular Operating Environment, MOE 2009) minimization feature. Next, the resulting ligand conformations were docked into the catalytic pocket of the available crystal structure of *Agaricus bisporus* tyrosinase, obtained from RCSB Protein Data Bank (http://www.rcsb.org).

As displayed in **Figure 4**, docking results for **1-5** revealed a common disposition within the catalytic residues, thus producing comparable binding modes. We expected that both, their structural similarity and the limited size of the binding pocket, mutually contribute in inducing the same alignment. In fact, the catalytic residues appear as a narrow hydrophobic funnel, with the metal Cu^{2+} cofactors located at the bottom of the cavity surrounding the active site. Structurally, the walls of the pocket are formed, from one side, by six histidines (His61, His85, His94, His259, His263, and His296),
which are involved in the coordination of two copper ions (Figure 4). The pocket is then completed
by the following residues: Gly86, Phe90, His244, Glu256, Asn260, Phe264, Arg268, Ser282, Val283,
Ala286, and Phe292.

313 Compounds 1–5 behaved similarly to that reported in Dong et al. (Dong, Liu, Liu, Yu & Cao, 2018), who investigated different series of TSCs, substituted on the C-N double bond. Specifically, docking 314 results showed that the sulfur atom of the thiourea group of all compounds is directed toward the 315 316 metal ions, positioning between the two metal cofactors. The resulting coordination bonds among sulfur atom and both copper ions thus ensure the formation of the ligand-protein complex. Such 317 interaction orientates the distal benzene ring in a narrow hydrophobic cleft formed by residues 318 Asn260, His263, Phe264, Arg268, Ser282, and Val283. The optimal disposition for a metal 319 coordination, combined with the engagement of the aromatic platform into the hydrophobic cavity, 320 would primarily contribute in forming the ligand-protein complex, and therefore in influencing the 321 potency of the inhibitor. However, other structural features could be invoked in determining / 322 affecting the inhibitory activities. 323

The active compounds 5 (Figure 4) and 2 (Figure S5B) bear a hydroxyl group at the *ortho* position. 324 325 Visual inspection of 2 in complex with tyrosinase (Figure S5B) revealed that the *ortho* hydroxyl 326 group is able to establish a hydrogen bond (donor) with the oxygen of the amide group of Met280. 327 This feature could presumably contribute to the activity, but it is not sufficient to ensure it. In fact, 4 328 (Figure S5A), which also carries an ortho hydroxyl group, is not active. An additional hydrogen bond interaction between the third hydroxyl group (absent in compound 4) in position 6 and the carbonyl 329 side chain of Asn260 participates in stabilizing 2 within the active pocket, thus contributing in its 330 331 moderate inhibitory activity. An analysis of the docking results of the most active compound 5 seems to suggest that many features would mutually concur to explain its highest tyrosinase inhibitory 332 activity. They include: a) the above-mentioned hydrogen bond between the ortho hydroxyl group and 333

the amide carbonyl of Met280, b) an arene-arene stacking interaction between the benzene ring and 334 335 the imidazole ring of the His263, and moreover c) the smaller size of the unsubstituted thioamide group, that allows it a deeper arrangement of the sulfur atom between the metal cofactors. The best 336 docking poses of the compounds with an ethyl substitution on the terminal nitrogen of the thiourea 337 group showed, in fact, that their sulfur atoms are slightly away from the straight line directed between 338 the metal cofactors. This displacement is due to the N-substitution, which forces the entire molecule, 339 340 also hindering the formation of the arene-arene stacking between the catechol ring and the imidazole ring of His263. This is particularly evident for compound 4 (Figure S5A), the N-ethyl substituted 341 derivative of 5, which is inactive. 342

Compounds 1 and 3 (Figures S5C and S5D, respectively), lacking of the *ortho* hydroxyl group, did not show any significant additional interactions. The predicted binding mode for compound 1 only indicates an arene-arene stacking interaction with the imidazole ring of His263, which orientates the three hydroxyl groups of the benzene ring towards a hydrophobic region of the enzymatic pocket.

347

348 *3.5 Cellular assays*

None of the compounds showed cytotoxicity (**Figure S6**) nor ability to stimulate cells to secrete IL-6 (**Figure S7**). IL-6 secretion expressed as ratio to viable cells in each condition was similar between control cells and cells treated with **1** - **5**. The lack of a significant impact of any of these compounds, although tested at the highest concentration, on the viability of human fibroblasts, that do not express tyrosinase, excludes their non-specific toxicity towards human cells. Similarly, data on IL-6 secretion of human fibroblasts upon incubation with **1** - **5** indicate that they do not activate cells in a proinflammatory fashion either.

356

357 **4. Conclusions**

Major part of tyrosinase inhibitors are polyphenolic compounds or thiourea derivatives (Pillaiyar, 358 359 Manickam & Namasivayam, 2017). TSCs 1-5 combine some relevant chemical characters of both the classes: the polyhydroxyl unit and the C=S group. Effectively, 1, 2, and 5 are inhibitors of 360 mushroom tyrosinase, and in particular 5 has an inhibitory activity stronger than kojic acid used as 361 reference. Compounds 1, 2, and 5 are mixed-type inhibitors just as kojic acid (Deri et al., 2016). These 362 compounds are evidently able to coordinate Cu(II) ions, as predicted by molecular modelling, and as 363 364 confirmed by potentiometric studies, but care has to be devoted not to oversimplify the inhibition mechanism overstressing this aspect: subtle differences in the structure of the molecule, such there 365 are between 5 and 4, or 1 and 3, change drastically the inhibition power, even if they do not alter 366 367 sensibly the complexation ability. Another relevant point to note is that the inhibitor activity is lost 368 when the NH₂ group of compound **5** is alkylated as in **4**, and the same happens in thiourea derivatives (Thanigaimalai et al., 2010). 369

Obviously, if it possible to think about practical applications of these tyrosinase inhibitors, they have to be safe. Compounds 1-5 resulted non-toxic on human fibroblasts and they did not induce a proinflammatory response in cells. These data and others in the literature (Xu, Liu, Zhu, Yu & Cao 2017; Song et al. 2017) are encouraging in considering TSCs tyrosinase inhibitors suitable for practical applications.

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376 Acknowledgements

377 "Centro Interdipartimentale Misure Giuseppe Casnati" of the University of Parma is thanked for378 facilities. The authors also thank Andrea Brancale for the use of the MOE program.

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