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Original

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; Fiscaro, 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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note finali coverpage

(Article begins on next page)

02 May 2026

1 **Hydroxyphenyl thiosemicarbazones as inhibitors of mushroom tyrosinase and**
2 **antibrowning agents**

3 M. Carcelli,^a D. Rogolino,^a J. Bartoli,^a N. Pala,^b C. Compari,^c N. Ronda,^c F. Bacciottini,^c M.
4 Incerti,^c E. Fisicaro^c

5

6 ^aDepartment of Chemistry, Life Sciences and Environmental Sustainability and CIRCMSB
7 (Consorzio Interuniversitario di Ricerca in Chimica dei Metalli nei Sistemi Biologici) Parma Unit,
8 University of Parma, Parco Area delle Scienze 17/A, 43124 Parma, Italy

9 ^bDepartment of Chemistry and Pharmacy, University of Sassari, Via Vienna 2, 07100 Sassari, Italy

10 ^cFood and Drug Department, University of Parma, Parco Area delle Scienze 27/A, 43124 Parma,
11 Italy

12

13

14 **Corresponding Author:**

15 Prof. Mauro Carcelli

16 Department of Chemistry, Life Sciences and Environmental Sustainability

17 University of Parma, Parco Area delle Scienze 17/A, 43124 Parma, Italy

18 phone: +390521905427

19 email: mauro.carcelli@unipr.it

20

21

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 *o*-
27 diphenols to *o*-quinones, with formation of brown or black pigments (melanines). Tyrosinase
28 inhibitors are of great interest in medicine and cosmetics (skin whitening compounds), but also in
29 food and beverage industry (antibrowning agents). Here we report on the activity as mushroom
30 tyrosinase inhibitors of a series of hydroxyphenyl thiosemicarbazones (**1-5**): one of them revealed an
31 inhibitory activity stronger than kojic acid, used as reference. Enzymatic inhibition activity was
32 confirmed by colorimetric measurements on small wheels of Fuji apples treated with the
33 hydroxyphenyl thiosemicarbazones. The mechanism of action of compounds **1-5** was investigated by
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
36 cytotoxic and they do not activate cells in a pro-inflammatory way.

37

38

39 **1. Introduction**

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

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

54 A large number of tyrosinase inhibitors have already been reported in the literature, from both
55 synthetic and natural sources (Zolghadri et al., 2019; Pillaiyar, Namasivayam, Manickam & Jung,
56 2018; Chan, Huang, Lee & Lin, 2014; Ullah, Son, Young Yun, Hyun Kim, Chun & Moon, 2016;
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
60 that only a few of available studies are well sounded from chemical and mechanistic point of view
61 (Tang, Cui, Li, Huang & Li, 2018; Xu, Liu, Zhu, Yu & Cao, 2017). Thiosemicarbazones (TSCs) have
62 a lot of different biological activities, and they have been also proposed as inhibitors of mushroom
63 tyrosinase (Yi et al. 2011; Xu, Liu, Zhu, Yu & Cao, 2017; Chen et al., 2012; Haldys et al., 2018).

64 Metalloenzymes could be inhibited by complexation of the metal ion/ions in their active site; in other
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
68 has a good affinity for the copper ions, and tyrosinase, that belongs to the Type-3 copper protein

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

84 All reagents were of analytical grade and were used as received. The purity of the synthesized
85 compounds was verified ≥ 95 % by elemental analysis. Technical data about NMR, ATR-IR,
86 electrospray mass spectral analyses (ESI-MS), and ICP analyses were already reported (Rogolino et
87 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

93 *in vacuum*. The precipitate was filtered off, washed with cold ethanol and dried in vacuum.
94 Characterizations (¹H-NMR, IR, MS, elemental analysis) of the TSCs **1-5** are reported in the
95 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,
100 Shimoishi, Tada & Takahata, 2003) with minor modifications, using L-DOPA (Sigma-Aldrich) as
101 substrate. Enzyme activity was determined at room temperature following the increase in absorbance
102 at 475 nm ($\epsilon = 3700 \text{ M}^{-1} \text{ cm}^{-1}$) determined by the formation of dopachrome by a Kontron Uvikon 860
103 spectrophotometer. Stock solutions of L-DOPA (15 mM), inhibitor (0.5 mM) and enzyme (2.5 U/ μ l)
104 were prepared in Na₂HPO₄-NaH₂PO₄ 25 mM buffer (pH 6.8) and then diluted to the appropriated test
105 concentrations. The experiments were performed at a constant enzyme concentration (12.5 U/mL),
106 varying inhibitor and substrate concentrations. The substrate was added at the last moment to the
107 reaction mixture and immediately absorbance measurement started for measuring the initial reaction
108 rate from the slope of the linear increase of optical density. 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-
109 4-one (kojic acid, Sigma-Aldrich) was used as positive control.

110

111 *2.4 Colorimetric measurements*

112 The colorimetric measurements were performed using Fuji apples, following the method of Wu *et al.*
113 (Wu, Cheng, Li, Wang & Ye, 2008). Apples were washed and cut into small wheels by a mandolin
114 slicer. After that, they were dipped in 200 mL of 30 μ M test solution, prepared in Na₂HPO₄-NaH₂PO₄
115 25 mM buffer (pH 6.8) for 3 min and then drained. This procedure was repeated for the compounds
116 **1, 2, 3** and **5**. Control samples were dipped in Na₂HPO₄-NaH₂PO₄ 25 mM buffer alone. Samples were

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

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

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

126

127 *2.5 Potentiometry*

128 The Cu(II) stock solution was prepared from $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck). Its concentration was determined
129 by titrating the solution with EDTA in presence of concentrate ammonia; Fast Sulfon Black is used
130 as indicator. Equilibrium constants at 25 ± 0.1 °C for protonation and complexation reactions were
131 determined as detailed in Rogolino et al., 2015. Temperature was controlled to ± 0.1 °C by a ISCO
132 GTR 2000 IIX bath. Appropriate amounts of ligand solution, prepared by weight, were titrated with
133 standard KOH or HCl (when starting from the alkaline solution $\text{pH} \approx 11$), with and without metal ions,
134 under magnetic stirring. The protonation constants were obtained by titrating 20 or 50 ml of samples
135 of each ligand (0.05 mmol). The speciation was defined by performing the titrations at different
136 ligand/metal ratios (1 up to 4). At least two measurements (about 60 experimental points each) were
137 performed for each system. The electrodic chain was calibrated, again, as detailed in Rogolino et al..
138 Speciation and the logarithm of the stability constants ($\log \beta_{\text{pqr}}$) are obtained by using the program
139 HYPERQUAD (Gans, Sabatini, & Vacca, 1996). In $\beta_{\text{pqr}} = [\text{M}_p\text{L}_q\text{H}_r]/[\text{M}]^p [\text{L}]^q [\text{H}]^r$, the equilibrium

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*

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

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 μM
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*

160 Molecular Operating Environment software package platform (MOE) was used for the current
161 computational study (Molecular Operating Environment, MOE 2009). A single database file
162 containing the ligands for molecular docking studies was prepared using MOE builder mask. The
163 geometries of the selected ligands were optimized by an energy minimization pass until a

164 convergence gradient criterion of $0.0001 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ 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 (<http://www.rcsb.org/>). 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

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

180

181 3. Results and discussion

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

187

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

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

$$207 \quad \frac{1}{v} = \left(1 + \frac{[I]}{K_i}\right) \left(\frac{K_m}{V_{max}}\right) * \frac{1}{[S]} + \left(1 + \frac{[I]}{\alpha K_i}\right) * \frac{1}{V_{max}}$$

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

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

214 Among the studied TSCs, only **5** possesses an inhibitory activity of practical interest, stronger than
215 those of the kojic acid assumed as reference, as revealed by the values of K_i and IC_{50} (**Table 1**). IC_{50}
216 is the concentration of the inhibitor able to halve the maximum reaction rate in the adopted
217 experimental conditions; the value reported for kojic acid agrees with the literature data, if normalized
218 for the tyrosinase concentration (Qamar et al. 2019; Sari, Barut, Özel, Kuruuzum-Uz & Şohretoğlu,
219 2019; Şöhretoğlua et al., 2018; Larik et al. 2017).

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

225

226 3.2 Antibrowning treatment of Fuji apple small wheels

227 The effects of the dipping in 30 μ M solutions of **1**, **2**, **3**, and **5** are qualitatively shown in **Figure 3**,
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
230 assay. The increase of browning follows, as expected, the reverse order of the inhibitory activity
231 against tyrosinase. The inactive compound **3**, as expected, behaves in the same way as the control
232 sample. On the contrary **5** seems to show the greatest antibrowning effect. It is much more difficult
233 to quantify the effect in terms of color differences by means of colorimetric measurements. Because
234 an increase of the parameter a^* is related with an increase in redness, it has been suggested (Abbott

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

243

244 3.3 Potentiometry

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

260 $pK_3=11.79$). Even if the different proton-dissociation equilibria are difficult to separate, for **4** and **5**,
261 the first dissociation constant can be reasonably attributed to one of the OH and the second one to the
262 dissociation of the N-H group of the thiosemicarbazide moiety (Enyedy et al. 2012). For **2** both the
263 constants pK_1 and pK_2 , very near as value, are probably attributable to two OH groups, and the third
264 one to the N-H proton; the presence of two ionizable substituents reduces the acidity of the N-H group
265 of about one order of magnitude.

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

278 The complexes of **4** are already present at acidic pH with formation of the neutral CuL species, having
279 a very high stability; at near pH = 8 CuL₂ begins to form. For a better comparison, we can consider
280 also **2** as a diprotic acid, setting L'= LH. With this substitution it is easy to see that the sets of
281 complexes formed in solution have the same stoichiometry for **2** and **4**, as expected, due to the
282 presence of the same chelating moiety. For **2** the insoluble species seem to be CuL' (i.e. CuLH),
283 corresponding to CuL for **4**. The distribution diagrams as a function of pH for L:Cu = 4:1 at a Cu(II)
284 concentration equal to 1.25 mM clearly show that formation of CuL (CuLH, in the case of **2**) starts at

285 low pH, confirming that TSCs are very efficient Cu(II) chelators (**Figure S4**) (Sendovski, Kanteev,
286 Ben-Yosef, Adir & Fishman, 2011). The chelation of the Cu(II) ion greatly increases the dissociation
287 of the hydrazine group in the complexes of **2** and **4**, particularly for **2**. The solubility of the Cu(II)
288 complexes follows a reverse order in comparison with the inhibitory activity, the complexes with **4**
289 (without inhibitory activity) being soluble, and those with **5** (the most potent inhibitor) completely
290 insoluble. Copper complexes of kojic acid are also completely insoluble in the full range of pH, not
291 allowing to be studied in solution (Bhatia, Kociok-Kohn & Molloy, 2015).

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

296

297 *3.4 Molecular modelling*

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

304 As displayed in **Figure 4**, docking results for **1-5** revealed a common disposition within the catalytic
305 residues, thus producing comparable binding modes. We expected that both, their structural similarity
306 and the limited size of the binding pocket, mutually contribute in inducing the same alignment. In
307 fact, the catalytic residues appear as a narrow hydrophobic funnel, with the metal Cu²⁺ cofactors
308 located at the bottom of the cavity surrounding the active site. Structurally, the walls of the pocket

309 are formed, from one side, by six histidines (His61, His85, His94, His259, His263, and His296),
310 which are involved in the coordination of two copper ions (**Figure 4**). The pocket is then completed
311 by the following residues: Gly86, Phe90, His244, Glu256, Asn260, Phe264, Arg268, Ser282, Val283,
312 Ala286, and Phe292.

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

324 The active compounds **5** (**Figure 4**) and **2** (**Figure S5B**) bear a hydroxyl group at the *ortho* position.
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
329 interaction between the third hydroxyl group (absent in compound **4**) in position 6 and the carbonyl
330 side chain of Asn260 participates in stabilizing **2** within the active pocket, thus contributing in its
331 moderate inhibitory activity. An analysis of the docking results of the most active compound **5** seems
332 to suggest that many features would mutually concur to explain its highest tyrosinase inhibitory
333 activity. They include: a) the above-mentioned hydrogen bond between the *ortho* hydroxyl group and

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

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

347

348 3.5 Cellular assays

349 None of the compounds showed cytotoxicity (**Figure S6**) nor ability to stimulate cells to secrete IL-
350 6 (**Figure S7**). IL-6 secretion expressed as ratio to viable cells in each condition was similar between
351 control cells and cells treated with **1** - **5**. The lack of a significant impact of any of these compounds,
352 although tested at the highest concentration, on the viability of human fibroblasts, that do not express
353 tyrosinase, excludes their non-specific toxicity towards human cells. Similarly, data on IL-6 secretion
354 of human fibroblasts upon incubation with **1** - **5** indicate that they do not activate cells in a pro-
355 inflammatory fashion either.

356

357 4. Conclusions

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

370 Obviously, if it possible to think about practical applications of these tyrosinase inhibitors, they have
371 to be safe. Compounds **1-5** resulted non-toxic on human fibroblasts and they did not induce a
372 proinflammatory response in cells. These data and others in the literature (Xu, Liu, Zhu, Yu & Cao
373 2017; Song et al. 2017) are encouraging in considering TSCs tyrosinase inhibitors suitable for
374 practical applications.

375

376 **Acknowledgements**

377 “Centro Interdipartimentale Misura Giuseppe Casnati” of the University of Parma is thanked for
378 facilities. The authors also thank Andrea Brancale for the use of the MOE program.

379

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