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Original article

Refinement of arylthiosemicarbazone pharmacophore in inhibition of mushroom tyrosinase

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ABSTRACT

Melanin play a major role in human skin protection and their biosynthesis is vital. Due to their color, they contribute to the skin pigmentation. Tyrosinase is a key enzyme involved in the first stage of melanin biosynthesis, it catalyzes the transformation of tyrosine into L-dopaquinone. The aim of the present study was to study molecules able to inhibit tyrosinase to be used in treating depigmentation-related disorders. In this study, we targeted arylthiosemicarbazone analogs with the aim to contribute to the identification of the optimal aryl ring to be linked to the thiosemicarbazone moiety. The biological activity was evaluated on commercial mushroom tyrosinase which was purified prior use. The results demonstrated that several of our compounds (**1a**–**h**, **1j**, **1r** and **5**) had more potent inhibitory activities than kojic acid which was used as the reference inhibitor.

1. Introduction

Hyperpigmentation of skin is frequently linked to an increased activity of melanogenic enzymes, such as tyrosinase (EC 1.14.18.1) [1,2]. The latter plays a pivotal role in the biosynthesis of melanin and it is considered to be the key enzyme in skin coloration and food browning [3]. Tyrosinase, is a copper-containing enzyme, widely present in mammals, plants and fungi, it accepts many phenols and catechols as substrates [4,5]. It is involved in the transformation of L-tyrosine into dopaquinone which occurs through two steps: hydroxylation of L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA), then oxidation of the latter to an *ortho*-quinone (dopaquinone). Dopaquinone is further transformed through several reactions to yield brown to black melanin which is responsible for color of mammals-skin [6].

Inhibitors/activators of tyrosinase became increasingly important in medicinal and cosmetic products. Tyrosinase inhibitors are used in drugs and cosmetics, directed for skin depigmentation [7–10], whereas compounds that increase melanogenesis such as tyrosinase activators may protect human skin from UV irradiation damage [11]. In this context, an increasing number of natural and synthetic compounds acting as tyrosinase inhibitors were reported [12], but only few of them are used as skin-whitening agents due to safety concerns.

Among reported tyrosinase inhibitors, thiourea derivatives have been particularly investigated [13,14]. Based on the potential of the thiourea motif for the inhibitory activity of tyrosinase, several recent studies have disclosed that thiosemicarbazones derivatives as potent inhibitors of tyrosinase [15–17]. One of us was among the first to highlight the importance of such compounds as inhibitors of melanogenesis [18–22]. More recently, Lee and co-workers have investigated structure–activity relationship of substituted phenylthiosemicarbazones as melanogenesis inhibitors [23,24]. They found that the hydrophobicity of the group attached on the thiosemicarbazone moiety is a determinant factor for the inhibitory activity in melanogenesis.

N-Phenylthiourea (PTU) was also shown to inhibit catechol oxidase enzyme that belongs to the type-3 copper-containing proteins. The crystal structure of catechol oxidase with PTU inhibitor reveals that the S-atom of the PTU binds to both copper ions in the active site of catechol oxidase and blocks enzyme activity

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[2] Equal senior contribution.

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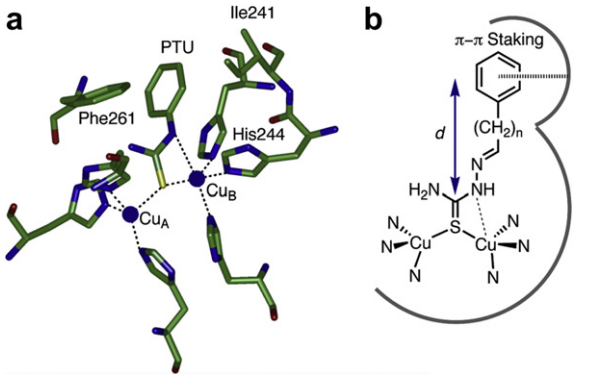


Fig. 1. (a) Crystal structure of the active site region of catechol oxidase from sweet potatoes (*Ipomoea batatas*) showing PTU bound to the dicopper center. The sulfur of the TBU binds to both copper ions and the hydrophobic cavity formed by residues Ile241, His244 and Phe261 provides van der Waals contacts with the aromatic ring of the TBU; (b) Putative binding mode of arylthiosemicarbazone derivatives showing the importance of the Cu–Cu bridging semicarbazone group, the π–π stacking of aryl group and the distance between the thiosemicarbazone and aryl moieties.

[25,26]. In addition, van der Waals interactions between the Phe261, Ile241, His244 residues of the hydrophobic cavity must contribute to the high affinity of the PTU to the enzyme (Fig. 1a). Although the crystal structure of mushroom tyrosinase is not yet known, the binding mode of PTU like compounds should be very much similar, since the active site of tyrosinase is highly conserved among different species. On the other hand, several investigations pointed out to the importance of the presence of an aromatic nucleus close to the chelating thiosemicarbazone moieties for good inhibition of mushroom tyrosinase [20–22].

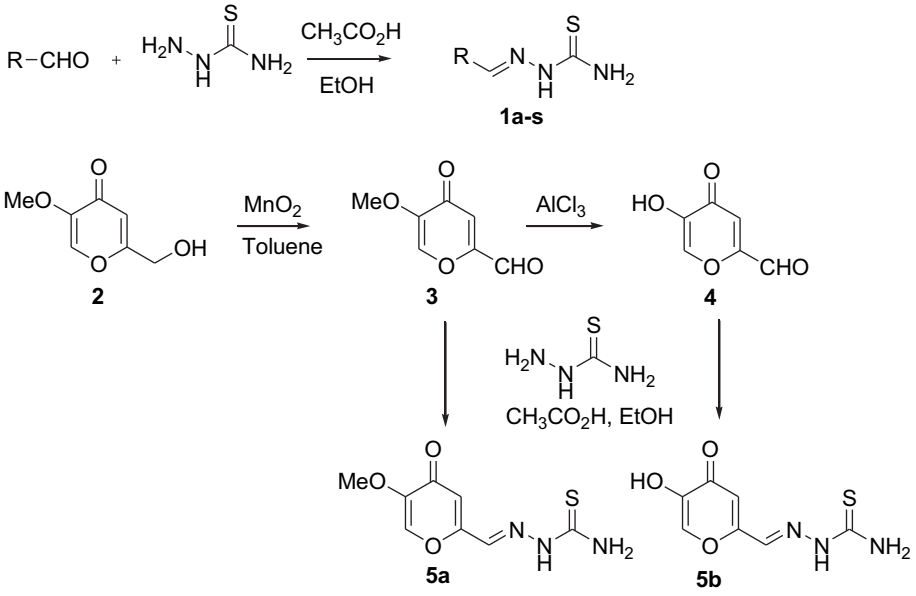
Taking advantage of above information and in continuing our program aimed to search for small molecules with a high potential effect as tyrosinase inhibitors [27,28], we targeted thiosemicarbazone pharmacophore. The aim was to optimize the structure of the aromatic moiety linked to the thiosemicarbazone moieties as well as the linker separating them (Fig. 1b). Herein, we report, the synthesis and the evaluation of the mushroom tyrosinase inhibition [29] of 19 new thiosemicarbazones compounds, **1a**–**s** and **5a**,**b**.

2. Results and discussion

The access to thiosemicarbazone derivatives **1a**–**s** and **5a**,**b** is outlined in Scheme 1. The syntheses of compounds **1a**–**s** involve condensation reactions of a carboxaldehyde with the thiosemicarbazide in anhydrous ethanol with acetic acid as the catalyst. For the preparation of compounds **5a**,**b**, commercially available kojic acid was first transformed into compound **2** according to already published method [30]. The oxidation of the latter with MnO_2 led to aldehyde **3** [31]. Demethylation of aldehyde **3** by $AlCl_3$ at 120 °C afforded the derivative **4**. A similar procedure to the synthesis of compounds **1a**–**s** was followed to obtain the compound **5a**,**b** in good yields from aldehydes **3** and **4**.

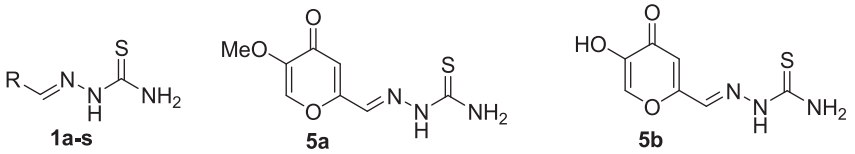
All the thiosemicarbazones **1a**–**s** and **5a**,**b** were evaluated on purified mushroom tyrosinase. In a typical experiment, preincubated inhibitors (20 μM, 0.1 mM, 0.2 mM, 1 mM and 2 mM) with 6 units of purified mushroom tyrosinase in phosphate buffer (50 mM, pH 7.0) were assayed through determination of L-DOPA (1.4 mM) oxidation. All compounds exhibited dose-dependent, inhibitory effect on the diphenolase activity of mushroom tyrosinase. Extend of inhibition was expressed as the inhibitor concentration leading to 50% loss of enzyme activity (IC_{50}). Values of IC_{50} are reported in Table 1. Kojic acid (KA) which exhibited an inhibition activity with an $IC_{50} = 0.102$ mM was used as reference. In our conditions, we found that the parent compound **1a** [23,24] was a better inhibitor than KA (9 μM vs. 102 μM). This value ranks this compound among the best inhibitors of mushroom tyrosinase.

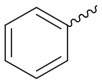
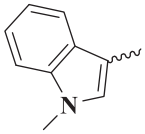
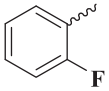
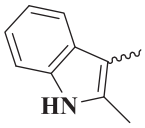
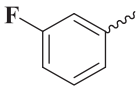
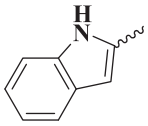
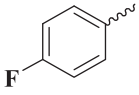
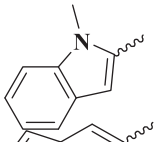
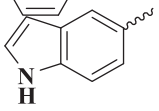
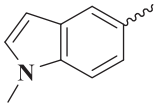
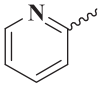
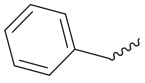
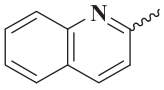
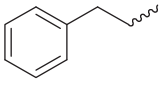
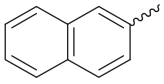
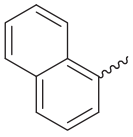
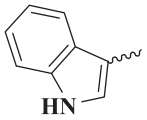
Around this structure, we then investigated which modifications could improve the IC_{50} of the thiosemicarbazone derivatives. We first studied the influence of the distance between the thiosemicarbazone moiety and the aromatic nucleus. While the addition of a methylene ($-CH_2-$) group did slightly enhance the inhibitory activity (**1a**: 9 μM vs. **1r**: 7 μM), the introduction of an ethylene group ($-CH_2-CH_2-$) led to a dramatic drop of activity (**1a**: 9 μM vs. **1s**: 400 μM). These results indicate that the distance separating the thiosemicarbazone group from the aromatic ring is critical for activity. This behavior was already described with thiourea compounds as inhibitors of melanogenesis in melanoma B16 cells. This effect was attributed to an increase of bulkiness of sp^3 carbons which prevent both the accessibility of thiourea compounds and π–π stacking of phenyl group [24].



Scheme 1. Synthesis of compounds **1a**–**s** and **5a**,**b**.

Table 1
Structures and inhibitory activities of derivatives **1a**–**s** and **5a**,**b**.



Compounds	R	IC_{50} (μM)	Compounds	R	IC_{50} (μM)
1a[a]		9 ± 1	**1l**		>2000
1b		32 ± 3	**1m**		>2000
1c		9 ± 1	**1n**		402 ± 50
1d		11 ± 1	**1o**		1700 ± 100
1e		6 ± 1	**1p**		>2000
1f		8 ± 1	**1q**		>2000
1g		45 ± 3	**1r**		7 ± 1
1h		70 ± 10	**1s**		400 ± 50
1i		1100 ± 100	**5a**	–	>2000
1j[b]		12 ± 2	**5b**	–	11 ± 2
1k		600 ± 100			

[a] Reported in Refs. [23,24].
[b] Reported in Ref. [24].

We then studied the effect of heterocycle rings such as 2-pyridyl, 2-pyrolidinyl or 2-furanyl rings instead of the phenyl group. Introduction of a 2-pyridyl substituent such as in **1g** led to a much less active analog (five folds increase of the IC_{50}) compared to the parent compound **1a**, while the introduction of 2-pyrolidinyl or 2-furanyl substituents provided compounds **1f** and **1e** with slightly better activity compared to **1a**. In our enzymatic conditions and at pH = 7, the pyridine nucleus ($pK_a = 5$) in **1g** is partially protonated. To the contrary, the pyrolidinyl ($pK_a = 0.5$) and the furanyl moieties in **1f** and **1e**, respectively, are not protonated. These results point out the importance of avoiding the presence of charged substituent at the phenyl group.

The next stage of our investigation was to check the size-effect of the aromatic ring linked to the thiosemicarbazone moiety. The size of the aromatic ring is critical for activity. In the pyrolidinyl series (**1f** vs. **1k**–**q**), the activity is dramatically decreased with more bulky pyrolidinyl containing substituents, such as indolic substituents ($IC_{50} > 500$ μM vs. 8 μM for **1f**). From activities of compounds **1k**–**q**, it can be learned that unsubstituted N–H of the pyrolidinyl moiety is required for better inhibition activity (**1k** vs. **1l** and **1n** vs. **1o**). The activity may depend on the position where the thiosemicarbazone moiety is linked (**1k**, **1n** and **1p**). The later effect was confirmed among the naphthyl series (compounds **1i**,**j**). The β-naphthyl derivative **1i** is a very weak inhibitor with an IC_{50} up to 1 mM while the α-naphthyl isomer **1j** [24] exhibits an IC_{50} close to the one of the parent compound **1a** ($IC_{50} = 12$ μM). The difference in activity between **1i** and **1j** may be due to sterical and conformational factors. The overall shape of **1i** is linear whereas **1j** is curved, assuming that **1j** may have the adequate shape for better fitting in the active site of the enzyme. Compound **1j** [24] was described as better inhibitor of melanogenesis in melanoma B16 cells than the parent compound **1a** (1.1 μM vs. >10 μM) [23]. These authors attributed this behavior to the high hydrophobicity of the naphthyl group compared to the phenyl one, which allows a better transfer across the membrane and a better biodisponibility of the inhibitor. In order to increase the hydrophobicity of the aromatic nucleus without increasing its size too much, we introduced fluorine atom at the *ortho*, *meta* and *para* position on the aromatic ring (compounds **1b**, **1c** and **1f**). These substitutions barely affected compound activity.

Finally, we studied the KA derivatives **5a**,**b** in which the KA moiety and the thiosemicarbazone are combined. We found that this modification enhanced considerably the inhibition activity of the KA. Indeed, compound **5b** is 9 times more active than its parent compound, KA. Since KA is supposed to bind the dicopper center by its α-hydro ketone group [32], we can expect that compound **5b** could be involved in two types of binding as depicted in Fig. 2. Because the methylation of the hydroxyl group (**5a**) has a negative effect on the inhibitory activity, we can assume that the compound **5b** binds to the dicopper center by its α-hydro ketone group (Fig. 2b).

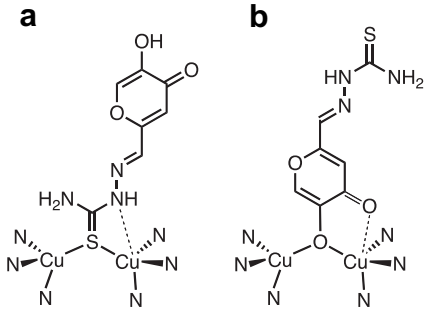


Fig. 2. The two putative binding modes for the kojic acid derivative **5b**.

3. Conclusion

In summary, the data provided in this report were proved to be informative about the structural requirement for developing new inhibitors for mushroom tyrosinase. Our results confirm the importance of the presence of an aromatic nucleus or heterocycles (furanyl or pyrolidyl) linked to the thiosemicarbazone pharmacophore. The toxicity of the most active compounds (**1e**, **1f**, **1r** and **5b**) was assessed through an MTT assay on two cell lines and found non toxic at concentrations higher than 1 mM (results not shown). Combined with recent literature data, our results will be of valuable help in the design of elegant and clinically useful tyrosinase inhibitors.

4. Experimental

4.1. Chemistry

^1H and ^{13}C NMR spectra were recorded on a Brüker Advance-400 instrument (400 MHz for ^1H, 100 MHz for ^{13}C). Chemical shifts (δ) are reported in ppm relative to Me_4Si as an internal standard. ESI mass spectra and elemental analyses were performed at the analysis facilities of the department of chemistry of the University of Grenoble, France. Thin-layer chromatography (TLC) was carried out using Merck silica gel F-254 plates (0.25 mm thick). Flash chromatography was carried out using Merck silica gel 60, 200–400 mesh. All solvents were distilled prior to use. Chemicals and reagents were obtained either from Aldrich or Acros companies and used as obtained.

4.1.1. General procedures for the synthesis of thiosemicarbazone derivatives *1a*–*s*

The appropriate aldehyde (1.1 mmol) was dissolved in anhydrous ethanol (10 mL), thiosemicarbazide (1.0 mmol) and acetic acid (0.2 mL) were added to the above solution. The reaction mixture was refluxed for 10 h and then was cooled to room temperature. The appearing precipitate was filtered, washed by ether and recrystallized from ethanol to obtain the corresponding 1-(1-arylethylidene)thiosemicarbazone compounds **1a**–**s**.

4.1.2. 1-Benzylidenethiosemicarbazone (*1a*)

Yield 73%; m.p. 162–163 °C; ^1H NMR (DMSO-d_6) δ: 11.44 (s, 1H, NH), 8.22 (br s, 1H, NH_2), 8.05 (s, 1H, CH), 8.00 (br s, 1H, NH_2), 7.79 (m, 2H, ArH), 7.39 (m, 3H, ArH); ^{13}C NMR (DMSO-d_6) δ: 180.0, 144.2, 136.2, 131.8, 130.7129.3; MS (ESI) 180 $(M + H)^+$, 202 $(M + Na)^+$; Anal. Calcd for $C_8H_9N_3S \cdot 0.5H_2O$: C, 51.06; H, 5.31, N, 22.34; found: C, 50.97; H, 5.07; N, 22.47. This compound was previously reported in Ref. [23,24].

4.1.3. 1-[(2-Fluorophenyl)methylene]thiosemicarbazone (*1b*)

Yield 81%; m.p. 183–184 °C; ^1H NMR (DMSO-d_6) δ: 11.57 (s, 1H, NH), 8.30 (br s, 1H, NH_2), 8.28 (s, 1H, CH), 8.25 (d, $J = 8.0$ Hz, 1H, ArH), 8.09 (br s, 1H, NH_2), 7.45 (m, 1H, ArH), 7.25 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6) δ: 180.1, 164.1, 161.6, 136.7, 133.8, 133.7, 128.9, 126.7, 123.8, 117.9; MS (ESI) 198 $(M + H)^+$, 220 $(M + Na)^+$; Anal. Calcd for $C_8H_8FN_3S$: C, 48.72; H, 4.09, N, 21.31; found: C, 48.55; H, 4.18; N, 21.12.

4.1.4. 1-[(3-Fluorophenyl)methylene]thiosemicarbazone (*1c*)

Yield 88%; m.p. 189–190 °C; ^1H NMR (DMSO-d_6) δ: 11.53 (s, 1H, NH), 8.28 (br s, 1H, NH_2), 8.17 (br s, 1H, NH_2), 8.03 (s, 1H, CH), 7.85 (d, $J = 8.8$ Hz, 1H, ArH), 7.52 (d, $J = 7.6$ Hz, 1H, ArH), 7.44 (m, 1H, ArH), 7.21 (m, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ: 180.2, 166.8, 163.3, 142.6, 139.0, 138.9, 132.7, 132.6, 126.3, 118.7, 118.5, 114.8, 114.6; MS

(ESI) 198 $(M + H)^+$, 220 $(M + Na)^+$; Anal. Calcd for $C_8H_8FN_3S$: C, 48.72; H, 4.09, N, 21.31; found: C, 48.61; H, 4.19; N, 21.66.

*4.1.5. 1-[(4-Fluorophenyl)methylene]thiosemicarbazone (**1d**)*

Yield 85%; m.p. 197–198 °C; ^{1}H NMR (DMSO-d_6) δ: 11.45 (s, 1H, NH), 8.22 (br s, 1H, NH_2), 8.05 (br s, 1H, NH_2), 8.03 (s, 1H, CH), 7.87 (m, 2H, ArH), 7.23 (t, J = 8.8 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6) δ: 180.0, 166.2, 163.8, 143.0, 132.9, 132.8, 131.6, 131.5, 117.8, 117.6; MS (ESI) 198 $(M + H)^+$, 220 $(M + Na)^+$; Anal. Calcd for $C_8H_8FN_3S$: C, 48.72; H, 4.09, N, 21.31; found: C, 48.99; H, 4.11; N, 21.47.

*4.1.6. 1-(Furan-2-ylmethylene)thiosemicarbazone (**1e**)*

Yield 87%; m.p. 153–154 °C; ^{1}H NMR (DMSO-d_6) δ: 11.44 (s, 1H, NH), 8.23 (br s, 1H, NH_2), 7.96 (s, 1H, CH), 7.80 (s, 1H, ArH), 7.64 (br s, 1H, NH_2), 6.96 (d, J = 2.8 Hz, 1H, ArH), 6.61 (s, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ: 179.7, 151.4, 147.0, 134.5, 114.8, 114.3; MS (ESI) 170 $(M + H)^+$, 192 $(M + Na)^+$; Anal. Calcd for $C_6H_7N_3OS$: C, 42.59; H, 4.17, N, 24.83; found: C, 42.53; H, 4.49; N, 24.68.

*4.1.7. 1-[(1H-Pyrrol-2-yl)methylene]thiosemicarbazone (**1f**)*

Yield 77%; m.p. 200–201 °C; ^{1}H NMR (DMSO-d_6) δ: 11.35 (s, 1H, NH), 11.27 (s, 1H, NH), 8.08 (br s, 1H, NH_2), 7.98 (br s, 1H, NH_2), 7.82 (s, 1H, CH), 6.97 (d, J = 1.2 Hz, 1H, ArH), 6.30 (m, 1H, ArH), 6.10 (dd, J = 8.4 and 3.6 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ: 179.2, 135.7, 129.6, 123.8, 114.9, 111.2; MS (ESI) 169 $(M + H)^+$, 191 $(M + Na)^+$; Anal. Calcd for $C_6H_8N_4S$: C, 42.84; H, 4.80, N, 33.31; found: C, 42.73; H, 5.14; N, 33.24.

*4.1.8. 1-(Pyridin-2-ylmethylene)thiosemicarbazone (**1g**)*

Yield 81%; m.p. 215–216 °C; ^{1}H NMR (DMSO-d_6) δ: 11.65 (s, 1H, NH), 8.55(d, J = 8.4 Hz, 1H, ArH), 8.36 (br s, 1H, NH_2), 8.28 (d, J = 8.4 Hz, 1H, ArH), 8.18 (br s, 1H, NH_2), 8.08 (s, 1H, CH), 7.82 (t, J = 8.4 Hz, 1H, ArH), 7.36 (t, J = 8.4 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ: 180.4, 155.3, 151.3, 144.5, 138.5, 126.1, 122.2; MS (ESI) 203 $(M + Na)^+$.

*4.1.9. 1-(Quinolin-2-ylmethylene)thiosemicarbazone (**1h**)*

Yield 88%; m.p. 242–243 °C; ^{1}H NMR (DMSO-d_6) δ: 11.83 (s, 1H, NH), 8.47 (br s, 1H, NH_2), 8.44 (d, J = 8.8 Hz, 1H, ArH), 8.35 (br s, 1H, NH_2), 8.44 (d, J = 7.6 Hz, 1H, ArH), 8.24 (s, 1H, CH), 7.99 (m, 2H, ArH), 7.76 (t, J = 7.6 Hz, 1H, ArH), 7.59 (d, J = 7.6 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ: 180.5, 155.9, 149.3, 144.6, 138.3, 132.0, 130.8, 129.9, 129.8, 129.2, 120.1; MS (ESI) 231$(M + H)^+$, 253 $(M + Na)^+$.

*4.1.10. 1-(Naphthalen-2-ylmethylene)thiosemicarbazone (**1i**)*

Yield 82%; m.p. 245–246 °C; ^{1}H NMR (DMSO-d_6) δ: 11.57 (s, 1H, NH), 8.30 (br s, 1H, NH_2), 8.23 (s, 1H, CH), 8.19 (d, J = 8.4 Hz, 1H, ArH), 8.13 (br s, 1H, NH_2), 8.11 (s, 1H, ArH), 8.00–7.85 (m, 3H, ArH), 7.54–7.52 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6) δ: 179.9, 144.3, 135.6, 134.8, 134.0, 130.9, 130.2, 129.7, 129.0, 128.7, 125.1; MS (ESI) 230 $(M + H)^+$, 252 $(M + Na)^+$.

*4.1.11. 1-(Naphthalen-1-ylmethylene)thiosemicarbazone (**1j**)*

Yield 87%; m.p. 233–234 °C; ^{1}H NMR (DMSO-d_6) δ: 11.49 (s, 1H, NH), 8.92 (s, 1H, CH), 8.35 (d, J = 8.4 Hz, 1H, ArH), 8.33 (s, 1H, NH_2), 8.23 (d, J = 7.9 Hz, 1H, ArH), 8.21 (s, 1H, NH_2), 7.99 (d, J = 8.4 Hz, 2H, ArH), 7.65 (dd, $J_1 = J_2$ = 7.9 Hz, 1H, ArH), 7.59–7.54 (d, J = 8.4 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6) δ: 179.9, 143.0, 135.4, 132.5, 132.3, 131.3, 130.9, 129.3, 128.2, 127.8, 127.6, 124.9; MS (ESI) 230 $(M + H)^+$, 252 $(M + Na)^+$; Anal. Calcd for $C_{12}H_{11}N_3S$: C, 62.86; H, 4.84, N, 18.33; found: C, 62.96; H, 5.00; N, 18.51. This compound was previously reported in Ref. [23].

*4.1.12. 1-[(1H-Indol-3-yl)methylene]thiosemicarbazone (**1k**)*

Yield 68%; m.p. 230–231 °C; ^{1}H NMR (DMSO-d_6) δ: 11.61 (s, 1H, NH), 11.20 (s, 1H, NH), 8.31 (s, 1H, CH), 8.23 (d, J = 8.0 Hz, 1H, ArH), 8.07 (br s, 1H, NH_2), 7.80 (s, 1H, ArH), 7.44 (br s, 1H, NH_2), 7.42 (d, J = 8.0 Hz, 1H, ArH), 7.20 (t, J = 7.6 Hz, 1H, ArH), 7.13 (t, J = 7.6 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ: 178.5, 142.9, 139.1, 133.1, 125.9, 124.7, 124.2, 122.7, 113.8, 113.2; MS (ESI) 219 $(M + H)^+$, 241 $(M + Na)^+$.

*4.1.13. 1-[(1-Methyl-indol-3-yl)methylene]thiosemicarbazone (**1l**)*

Yield 89%; m.p. 206–207 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.18 (s, 1H, NH), 8.28 (s, 1H, CH), 8.25 (d, J = 8.0 Hz, 1H, ArH), 8.07 (br s, 1H, NH_2), 7.79 (s, 1H, ArH), 7.48 (d, J = 8.0 Hz, 1H, ArH), 7.46 (br s, 1H, NH_2), 7.26 (m, 1H, ArH), 7.17 (m, 1H, ArH), 3.80 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 178.4, 142.4, 139.6, 136.7, 126.3, 124.7, 124.4, 122.9, 112.1, 34.8; MS (ESI) 233 $(M + H)^+$, 255 $(M + Na)^+$.

*4.1.14. 1-[(1-Methyl-1H-indol-3-yl)methylene]thiosemicarbazone (**1m**)*

Yield 78%; m.p. 211–212 °C; ^{1}H NMR (DMSO-d_6) δ: 11.50 (s, 1H, NH), 11.03 (s, 1H, NH), 8.42 (s, 1H, CH), 8.20 (br s, 1H, NH_2), 8.13 (d, J = 7.8 Hz, 1H, ArH), 8.04 (s, 1H, ArH), 8.02 (br s, 1H, NH_2), 7.86 (d, J = 8.4 Hz, 1H, ArH), 7.81 (d, J = 8.4 Hz, 1H, ArH), 7.19 (dd, J = 8.4 and 1.6 Hz, 1H, ArH), 3.89 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 178.1, 142.8, 142.7, 137.7, 127.0, 123.8, 123.4, 122.5, 112.8, 108.9, 13.5; MS (ESI) 233 $(M + H)^+$, 255 $(M + Na)^+$.

*4.1.15. 1-[(1H-Indol-2-yl)methylene]thiosemicarbazone (**1n**)*

Yield 72%; m.p. 231–231 °C; ^{1}H NMR (DMSO-d_6) δ: 11.59 (s, 1H, NH), 11.42 (s, 1H, NH), 8.34 (br s, 1H, NH_2), 8.19 (br s, 1H, NH_2), 8.01(s, 1H, CH), 7.55 (d, J = 8.0 Hz, 1H, ArH), 7.36 (d, J = 8.0 Hz, 1H, ArH), 7.17 (t, J = 8.0 Hz, 1H, ArH), 7.01 (t, J = 8.0 Hz, 1H, ArH), 6.77 (s, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ: 179.9, 139.1, 135.8, 135.2, 130.0, 125.5, 122.9, 121.5, 113.1, 108.1; MS (ESI) 219 $(M + H)^+$, 241 $(M + Na)^+$.

*4.1.16. 1-[(1-Methyl-indol-2-yl)methylene]thiosemicarbazone (**1o**)*

Yield 92%; m.p. 204–205 °C; ^{1}H NMR (DMSO-d_6) δ: 11.44 (s, 1H, NH), 8.28 (br s, 1H, NH_2), 8.25 (s, 1H, CH), 7.66 (br s, 1H, NH_2), 7.56 (d, J = 8.0 Hz, 1H, ArH), 7.50 (d, J = 8.0 Hz, 1H, ArH), 7.23–7.21 (m, 1H, ArH), 6.94 (s, 1H, ArH), 3.96 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 179.6, 141.2, 138.1, 135.3, 128.8, 125.3, 122.9, 121.9, 112.1, 109.0, 33.8; MS (ESI) 233 $(M + H)^+$, 255 $(M + Na)^+$.

*4.1.17. 1-[(1H-Indol-5-yl)methylene]thiosemicarbazone (**1p**)*

Yield 76%; m.p. 217–218 °C; ^{1}H NMR (DMSO-d_6) δ: 11.28 (s, 2H, NH), 8.14 (s, 1H, CH), 8.08 (br s, 1H, NH_2), 7.88 (br s, 1H, NH_2), 7.86 (s, 1H, ArH), 7.66 (d, J = 8.4 Hz, 1H, ArH), 7.41 (d, J = 8.4 Hz, 1H, ArH), 7.40 (s, 1H, ArH), 6.46 (s, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ: 179.3, 146.6, 138.9, 129.6, 128.4, 127.3, 123.1, 121.9, 113.8, 103.9; MS (ESI) 219 $(M + H)^+$, 241 $(M + Na)^+$.

*4.1.18. 1-[(1-Methyl-indol-5-yl)methylene]thiosemicarbazone (**1q**)*

Yield 90%; m.p. 215–216 °C; ^{1}H NMR (DMSO-d_6) δ: 11.31 (s, 1H, NH), 8.15 (s, 1H, CH), 8.11 (br s, 1H, NH_2), 7.92 (br s, 1H, NH_2), 7.87 (s, 1H, ArH), 7.73 (d, J = 8.4 Hz, 1H, ArH), 7.46 (d, J = 8.4 Hz, 1H, ArH), 7.36 (d, J = 3.2 Hz, 1H, ArH), 6.47 (d, J = 3.2 Hz, 1H, ArH), 3.82 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 179.4, 146.4, 139.3, 132.7, 129.9, 127.3, 123.3, 121.9, 112.2, 103.2, 34.7; MS (ESI) 233 $(M + H)^+$, 255 $(M + Na)^+$.

*4.1.19. 1-(2-Phenylethylidene)thiosemicarbazone (**1r**)*

Yield 81%; m.p. 163–164 °C; ^{1}H NMR (DMSO-d_6) δ: 11.17 (s, 1H, NH), 8.07 (br s, 1H, NH_2), 7.59 (br s, 1H, NH_2), 7.47 (s, 1H, CH), 7.33 (m, 2H, ArH), 7.28 (m, 3H, ArH), 3.52 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 179.8, 147.5, 138.7, 130.9, 130.7, 128.7, 40.1; MS (ESI) 194 $(M + H)^+$, 216 $(M + Na)^+$; Anal. Calcd for $C_9H_{11}N_3S$: C, 55.94; H, 5.74, N, 21.75; found: C, 55.49; H, 5.82; N, 21.40.

*4.1.20. 1-(3-Phenylpropylidene)thiosemicarbazone (**1s**)*

Yield 65%; m.p. 119–120 °C; ^{1}H NMR (DMSO-d_6) δ: 11.09 (s, 1H, NH), 7.99 (br s, 1H, NH_2), 7.48 (br s, 1H, NH_2), 7.44 (t, J = 4.8 Hz, 1H, CH), 7.30–7.16 (m, 5H, ArH), 2.80 (t, J = 8.0 Hz, 1H, CH_2), 2.51 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 179.5, 148.6, 143.1, 130.4, 128.0, 35.4, 33.6; MS (ESI) 208 $(M + H)^+$, 230 $(M + Na)^+$.

*4.1.21. 2-Formyl-5-methoxy-4H-pyran-4-one (**3**)*

2-(Hydroxymethyl)-5-methoxy-4*H*-pyran-4-one **2** (1.50 g, 9.6 mmol) was dissolved in boiling toluene (50 mL), then treated with manganese dioxide (8.7 g, 100.0 mmol) and the mixture was heated at 120 °C. After completion of the reaction as indicated by TLC, the solution was filtered. The solvent was evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel (EtOAc/cyclohexane, 1:2) to obtain **3** as a white solid: yield 82%; mp 202–203 °C; ^{1}H NMR (DMSO-d_6) δ: 9.63 (s, 1H), 8.31 (s, 1H), 7.10 (s, 1H), 3.37 (s, 3H).

*4.1.22. Comenaldehyde (**4**)*

2-Formyl-5-methoxy-4*H*-pyran-4-one **3** (1.0 g, 6.5 mmol) was mixed with sublimed $AlCl_3$ (3.0 g, 22.5 mmol) and heated at 120 °C for 3 h. The reaction was decomposed by addition of HCl (100 mL, 10%), and the aqueous solution was extracted continuously with EtOAc. The organic solution was evaporated under reduced pressure and the residue was purified by flash chromatography on silica gel (AcOEt/cyclohexane, 1:1) to provide **4** as a colorless solid: yield, 56%; mp 163–164 °C; ^{1}H NMR (DMSO-d_6) δ: 9.73 (s, 1H), 9.12 (s, 1H), 8.41 (s, 1H), 7.23 (s, 1H).

*4.1.23. 1-[(5-Methoxy-4-oxo-4H-pyran-2-yl)methylene] thiosemicarbazone (**5a**)*

To a solution of compound **3** (0.150 g, 0.66 mmol) in anhydrous ethanol (10 mL), thiosemicarbazide (0.048 g, 0.5 mmol) was added. The reaction mixture was refluxed for 24 h and cooled to room temperature. The precipitate solid was filtered, washed with ether, and purified by recrystallization from ethanol to afford compound **5a** as a yellow solid: Yield 87%; ^{1}H NMR (DMSO-d_6) δ: 9.31 (s, 1H), 8.53 (s, 1H), 8.11 (s, 1H), 8.04 (s, 1H), 7.77 (s, 1H), 7.20 (s, 1H); MS (ESI) 228 $(M + H)^+$; Anal. Calcd for $C_8H_9N_3O_3S$: C, 42.28; H, 3.99, N, 18.49; found: C, 42.44; H, 3.95; N, 18.43.

*4.1.24. 1-[(5-Hydroxy-4-oxo-4H-pyran-2-yl)methylene] thiosemicarbazone (**5b**)*

The title compound was prepared following the same procedure as for **5a**. Yield 80%; m.p. 269–270 °C; ^{1}H NMR (DMSO-d_6) δ: 11.81 (s, 1H), 9.31 (s, 1H), 8.50 (s, 1H), 8.19 (s, 1H), 8.06 (s, 1H), 7.73 (s, 1H), 7.17 (s, 1H); ^{13}C NMR (DMSO-d_6) δ: 180.7, 175.4, 160.7, 148.9, 141.4, 135.7, 112.3; MS (ESI) 212 $(M - H)^-$; Anal. Calcd for $C_7H_7N_3O_3S$: C, 39.44; H, 3.31, N, 19.71; found: C, 39.80; H, 3.50; N, 19.69.

4.2. Biological evaluation

Mushroom tyrosinase (6.4 mg) purchased from Sigma (St. Louis, MO, USA) was dissolved in 50 mM phosphate buffer (5 mL, pH 7.0) and purified on Q-sepharose FF chromatography by a gradient of NaCl from 0 to 1.0 M [33]. Purity of tyrosinase was checked with SDS-PAGE; the purified tyrosinase exhibits only two bands at M.W. = ca. 14 kDa and 45 kDa. The tyrosinase activity was checked with spectroscopic method using L-DOPA as substrate.

All compounds were dissolved in DMSO stock solution 10%. Phosphate buffer pH 7.0 was used to dilute the DMSO stock solution of the compounds. Six units of mushroom tyrosinase (6.4 μg/ml) was first pre-incubated with the compounds, in 50 mM phosphate buffer (pH 7.0) for 5 min at 25 °C. The L-DOPA (2 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm of the DOPAchrome for 5 min. IC_{50} value, the concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of dose–response curves.

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