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Short communication

A class of potent tyrosinase inhibitors: Alkylidenethiosemicarbazide compounds

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Abstract

A series of alkylidenethiosemicarbazide compounds were synthesized and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were evaluated. The results showed that most of the synthesized compounds exhibited significant inhibitory activities. Especially, compound **1f** was found to be the most potent inhibitor with IC_{50} value of 0.086 μM, suggesting that further development of such compounds may be of interest.

Keywords: Synthesis; Alkylidenethiosemicarbazide compounds; Tyrosinase inhibitor

1. Introduction

Tyrosinase (EC 1.14.18.1) is a multifunctional copper-containing enzyme which is widely distributed in plants and animals [1]. It is well known that tyrosinase can catalyze the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinone (diphenolase activity). And the enzymatic oxidation of L-tyrosine into melanin is of considerable importance in coloring of skin, hair and eyes, and in food browning [2,3]. In addition, tyrosinase is also related to the molting process of insects [4] and adhesion of marine organisms [3,5].

Nowadays, tyrosinase inhibitors are thought to be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation [6,7], and useful in cosmetic products and food industry [8]. So far, a large number of potential tyrosinase inhibitors have been discovered from natural, synthetic and even from semi-synthetic sources, such as hydroquinone [9], ascorbic acid derivates [10], kojic acid (Fig. 1) [11], azelaic acid [12], corticosteroids [12], retinoids [12], arbutin (Fig. 1) [12] and tropolone (Fig. 1) [13,14]. Among all the known tyrosinase inhibitors, tropolone ($IC_{50} = 0.4$ μM) was found to be one of the most potent tyrosinase inhibitors [14]. Unfortunately, only few of the reported compounds are used in medicinal and cosmetic products because of their lower activities or serious side effects. Therefore, it is still necessary to search and discover novel tyrosinase inhibitors with higher activity and lower side effect.

Previous literatures described that thiourea derivatives, such as phenylthioureas [15,16], alkylthioureas [17] and 1,3-bis-(5-methanesulfonylbutyl)thiourea, displayed weak or moderate tyrosinase inhibitory activity. More recently, our investigation [18] also demonstrated that 1-(1-arylethylidene)thiosemicarbzide derivatives exhibited potent inhibitory activities against mushroom tyrosinase. Stimulated by these results, in the present investigation, we designed and synthesized a series of novel alkylidenethiosemicarbazide compounds bearing various alkyl substituents. We report here the preparation of alkylidenethiosemicarbazide compounds and their inhibitory effects on the diphenolase activity of mushroom tyrosinase.

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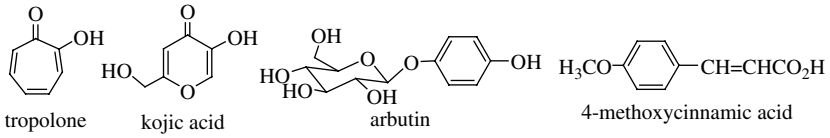


Fig. 1. Chemical structure of known tyrosinase inhibitors.

2. Chemistry

The alkylidenethiosemicarbazides were prepared by the condensation reaction of alkyl ketones or aldehydes with thiosemicarbazide in the presence of acetic acid in ethanol. To investigate the effects of thiosemicarbazido group on tyrosinase inhibitory activity, pentane-2,4-dione, hexane-2,5-dione, 2-acetylcyclohexanone and cyclohexane-1,3-dione were selected to prepare dithiosemicarbazide compounds. Unfortunately, only mono-thiosemicarbazido group products were obtained (Scheme 1).

3. Biology

Taking all the newly synthesized compounds as the effectors, we investigated their inhibitory effects on the diphenolase activity of mushroom tyrosinase. The IC_{50} value of these compounds are summarized in Table 1.

From the data shown in Table 1, the following conclusions were drawn.

(1) All the compounds exhibited potent inhibitory effects on the diphenolase activity of mushroom tyrosinase. Particularly, compounds **1a** ($IC_{50} = 0.23$ μM), **1b** ($IC_{50} = 0.20$ μM), **1f** ($IC_{50} = 0.086$ μM) and **1g** ($IC_{50} = 0.28$ μM) demonstrated more potent inhibitory activities than reference standard inhibitors arbutin (30% inhibition at 10.4 mM) and 3-(4-methoxyphenyl)acrylic acid ($IC_{50} = 0.41$ μM), and the most representative inhibitor, tropolone ($IC_{50} = 0.4$ μM), described by the literature [13,14].

(2) With the increase of the length of chain **R** (for 1-alkylidenethiosemicarbazides **1a–e** and 1-(1-alkylethylidene)thiosemicarbazides **1f–k**), the inhibitory activities decreased gradually. Interestingly, compound **1f** bearing a methyl group was found to be the most potent inhibitor with an IC_{50} value of 0.086 μM. These results suggested that the increase of the length of alkyl chain might cause stereohindrance for the inhibitors approaching the active site of the enzyme.

(3) Compound **2a** was 5.5-fold more active than compound **2b**, which indicated that the smaller size of the ring in volume would be beneficial to the molecules to approach the active centre of the enzyme. These results further confirmed that the shorter chain **R** was more favorable for the inhibitory activities of obtained compounds.

(4) The synthesized compounds with a saturated **R** substituents displayed stronger inhibitory activities than those compounds with an unsaturated **R** substituents, which suggested that the double bonds might be detrimental to their inhibitory activities.

(5) Compound **3** bearing a carbonyl group was also less active than the corresponding thiosemicarbazides without a carbonyl group. These results indicated that electron-withdrawing effects of the carbonyl group might play a vital role in determining their inhibitory activities.

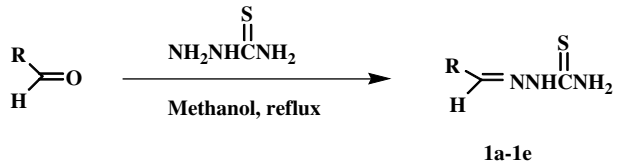
4. Conclusions

The present investigation reported that, for the first time, alkylidenethiosemicarbazide compounds had potent inhibitory effects on the diphenolase activity of mushroom tyrosinase. Interestingly, compound **1f** was found to be the most potent inhibitor with IC_{50} value of 0.086 μM. Preliminary structure–activity relationships' (SARs) analysis indicated that (1) the increase of the length of alkyl chain might cause stereohindrance for the inhibitors approaching the active site of the enzyme resulting in the decrease of inhibitory activities, and the shorter alkyl chain was more favorable; (2) the double bonds might be detrimental to their activities; and (3) the electron-withdrawing substituents might play a vital role in determining their inhibitory activities. These results suggested that further development of such compounds may be of interest.

5. Experimental protocols

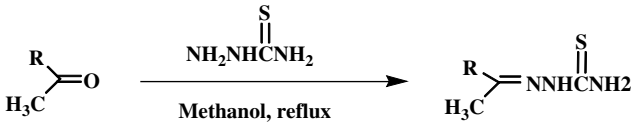
5.1. Reagents and general procedures

Melting points (m.p.) were determined with WRS-1B melting point apparatus and the thermometer was uncorrected. NMR spectra were recorded on Mercury-Plus 300 spectrometers at 25 °C in $CDCl_3$ or DMSO-d_6. All chemical shifts (δ) are quoted in parts per million downfield from TMS and coupling constants (J) are given in hertz. LC–MS spectra were recorded using the LCMS-2010A. All reactions were monitored by TLC (Merck Kieselgel 60 F_{254}) and the spots were visualized under UV light. Elemental analyses were performed on a Vario EL instrument and were within ±0.4% of the theoretical values. Infrared (IR) spectra were recorded on VECTOR 22 spectrometer. The appropriate aldehyde, ketone, thiosemicarbazide, and 4-methoxycinnamic acid were purchased from Darui Chemical Co. (ShangHai, China), arbutin was obtained from Brillian Biochemical Co. The other commercially available reagents and solvents were used without further purification. Mushroom tyrosinase (specific activity



1a-1e

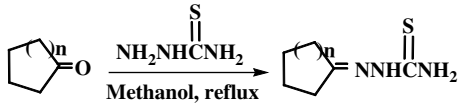
1a, R = CH_3; **1b**, R = C_2H5; **1c**, R = *Iso*-C_3H_7; **1d**, R = $CH_3CH{=}CH$; **1e**, R = $PhCH{=}CH$



1f-1j

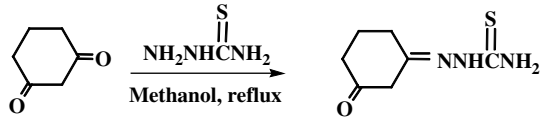
1f, R = CH_3; **1g**, R = C_2H_5; **1h**, R = $CH{=}C(CH_3)_2$; **1i**, R = 4-$CH_3OC_6H_4CH_2$;

1j, R^2 = 4-$HOC_6H_4CH_2CH_2$



2a - 2b

2a. n=1; **2b**. n=2



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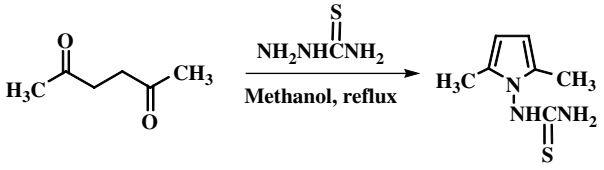

4

Scheme 1. Synthesis of alkylidenethiosemicarbazide compounds.

of the enzyme is 5360 U/mg) and L-DOPA (L-3,4-dihydroxyphenylalanine) were purchased from Sigma Chemical Co.

5.2. General procedures for the synthesis of alkylidenethiosemicarbazide compounds

The appropriate aldehyde or ketone (10 mmol) was dissolved in anhydrous ethanol (10 mL), and thiosemicarbazide (10 mmol) and acetic acid (0.5 mL) were added into the solution. After being refluxed for 24 h, the reaction mixture was cooled to room temperature and then the precipitate appeared. The corresponding pure compounds were obtained by filtration. For some special cases, the target compounds could be purified by recrystallization from ethanol.

5.2.1. 1-Ethylidenethiosemicarbazide (*1a*)

M.p. 135–136 °C. $R_f = 0.58$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.01 (1H, br s, NH), 7.90 (1H, br s, NH_2), 7.43 (1H, br s, NH_2), 7.37 (H, q, $J = 5.4$ Hz, CH), 1.84 (3H, d, $J = 5.4$ Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 177.9, 144.5, 19.0. IR (KBr): 3379, 3261, 3189, 3024, 1591, 1535 cm^{-1}. MS (ESI): *m/z* (100%) = 118 (M + 1). Anal. Calcd for $C_3H_7N_3S$ (117.17): C, 30.75; H, 6.02; N, 35.86. Found: C, 30.84; H, 5.99; N, 35.98.

5.2.2. 1-Propylidenethiosemicarbazide (*1b*)

M.p. 150–152 °C. $R_f = 0.62$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^{1}H NMR (300 MHz, DMSO-d_6):

Table 1
Tyrosinase inhibitory activities of alkylidenethiosemicarbazide compounds

Compounds	Yield (%)	IC_{50} (μM) (mean ± SEM[a])
1a	89.2	0.23 ± 0.015
1b	83.6	0.20 ± 0.018
1c	85.1	0.62 ± 0.05
1d	81.5	1.00 ± 0.026
1e	76.2	2.70 ± 0.058
1f	91.5	0.086 ± 0.009
1g	88.3	0.28 ± 0.023
1h	53.6	11.50 ± 0.13
1i	68.1	0.42 ± 0.031
1j	56.8	0.54 ± 0.012
2a	78.3	0.17 ± 0.011
2b	74.6	0.95 ± 0.032
3	51.0	15.10 ± 0.030
4	46.8	0.85 ± 0.064
4-Methoxycinnamic acid[b]		0.41 (mM) ± 0.005
Arbutin[c]		10.40 (mM) ± 0.089

[a] SEM: standard error of the mean.
[b] IC_{50} values in the literature is 0.34–0.43 mM [20,21].
[c] The concentration of 10.4 mM corresponding to inhibition percentage, determined in this work, is 30%. The reported IC_{50} value of arbutin was more than 30 mM [22].

δ 10.99 (1H, br s, NH), 7.89 (1H, br s, NH_2), 7.39 (1H, br s, NH_2), 7.38 (1H, t, $J = 3.8$ Hz, CH), 2.17–2.20 (2H, m, CH_2), 0.97 (3H, t, $J = 4.8$ Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 178.1, 149.0, 26.0, 11.3. IR (KBr): 3385, 3273, 3189, 3036, 1586, 1541 cm^{-1}. MS (ESI): *m/z* (100%) = 132 (M + 1). Anal. Calcd for $C_4H_9N_3S$ (131.20): C, 36.62; H, 6.91; N, 32.03. Found: C, 36.84; H, 6.97; N, 32.22.

5.2.3. 1-(3-Methylbutylidene)thiosemicarbazide (**1c**)

$R_f = 0.73$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, $CDCl_3$): δ 9.86 (1H, br s, NH), 7.28 (1H, br s, NH_2), 7.08 (1H, br s, NH_2), 6.48 (1H, t, $J = 6.3$ Hz, CH), 2.10 (2H, t, $J = 6.0$ Hz, CH_2), 1.88–1.91 (1H, m, CH), 0.97 (6H, d, $J = 6.3$ Hz, $2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.6, 148.8, 41.4, 26.9, 22.8. IR (KBr): 3425, 3282, 3144, 2951, 1590, 1537, 1383 cm^{-1}. MS (ESI): *m/z* (100%) = 160 (M + 1).

5.2.4. 1-(But-2-enylidene)thiosemicarbazide (**1d**)

M.p. 142–143 °C. $R_f = 0.73$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, $CDCl_3$): δ 9.70 (1H, br s, NH), 7.53 (1H, d, $J = 7.8$ Hz, CH), 7.06 (1H, br s, NH_2), 6.37 (1H, br s, NH_2), 6.13–6.18 (2H, m, 2CH), 1.91 (3H, d, $J = 4.5$ Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.2, 145.6, 138.9, 129.1, 19.3. IR (KBr): 3384, 3275, 3163, 3021, 2961, 1585, 1527 cm^{-1}. MS (ESI): *m/z* (100%) = 144 (M + 1). Anal. Calcd for $C_5H_9N_3S$ (143.21): C, 41.93; H, 6.33; N, 29.34. Found: C, 41.84; H, 6.18; N, 29.68.

5.2.5. 1-(3-Phenylallylidene)thiosemicarbazide (**1e**)

M.p. 134–136 °C. $R_f = 0.75$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, $CDCl_3$): δ 11.36 (1H, br s, NH), 8.15 (1H, br s, NH_2), 7.87 (1H, d, $J = 9.0$ Hz, CH), 7.58 (1H, br s, NH_2), 7.55 (2H, d, $J = 7.2$ Hz, phH), 7.32–7.37 (3H, m, phH), 7.03 (1H, d, $J = 16.2$ Hz, CH), 6.89 (1H, t, $J = 9.3$ Hz, CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 178.3, 145.4, 139.5, 136.5, 129.8, 129.5, 127.6, 125.7. IR (KBr): 3398, 3279, 3152, 3028, 1601, 757 cm^{-1}. MS (ESI): *m/z* (100%) = 206 (M + 1).

5.2.6. 1-(Propan-2-ylidene)thiosemicarbazide (**1f**)

M.p. 180–181 °C. $R_f = 0.55$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, DMSO-d_6): δ 9.86 (1H, br s, NH), 7.95 (1H, br s, NH_2), 7.48 (1H, br s, NH_2), 1.91 (3H, s, CH_3), 1.89 (3H, s, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 178.9, 152.3, 25.9, 18.4. IR (KBr): 3378, 3234, 3152, 2997, 1596, 1512 cm^{-1}. MS (ESI): *m/z* (100%) = 132 (M + 1). Anal. Calcd for $C_4H_9N_3S$ (131.20): C, 36.62; H, 6.91; N, 32.03. Found: C, 36.59; H, 6.84; N, 32.25.

5.2.7. 1-(Butan-2-ylidene)thiosemicarbazide (**1g**)

M.p. 98–100 °C. $R_f = 0.65$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, DMSO-d_6): δ 8.58 (1H, br s, NH), 7.26 (1H, br s, NH_2), 6.45 (1H, br s, NH_2), 2.31–2.33 (2H, m, CH_2), 1.90 (3H, s, CH_3), 1.10 (3H, t, $J = 7.6$ Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.1, 155.9, 32.3, 17.1, 11.4. IR (KBr): 3381, 3236, 3150, 3019, 2971, 1578 cm^{-1}. MS (ESI): *m/z* (100%) = 146 (M + 1). Anal. Calcd for $C_5H_{11}N_3S$ (145.23): C, 41.35; H, 7.63; N, 28.93. Found: C, 41.17; H, 7.55; N, 28.71.

5.2.8. 1-(4-Methylpent-3-en-2-ylidene) thiosemicarbazide (**1h**)

M.p. 119–120 °C. $R_f = 0.70$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, DMSO-d_6): δ 9.76 (1H, br s, NH), 8.09 (1H, br s, NH_2), 7.55 (1H, br s, NH_2), 4.74 (1H, s, CH), 1.94 (3H, s, CH_3), 0.99 (6H, s, $2CH_3$). ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.1, 153.5, 152.9, 136.7, 25.9, 20.1. IR (KBr): 3385, 3263, 3158, 3025, 2961, 1385 cm^{-1}. MS (ESI): *m/z* (100%) = 172 (M + 1).

5.2.9. 1-(1-(4-Methoxyphenyl)propan-2-ylidene)-thiosemicarbazide (**1i**)

M.p. 122–123 °C. $R_f = 0.68$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, $CDCl_3$): δ 8.50 (1H, br s, NH), 7.25 (1H, br s, NH_2), 7.09 (2H, d, $J = 8.7$ Hz, phH), 6.86 (2H, d, $J = 8.7$ Hz, phH), 6.34 (1H, br s, NH_2), 3.80 (3H, s, CH_3), 3.50 (2H, s, CH_2), 1.81 (3H, s, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 180.2, 157.6, 155.7, 131.2, 130.5, 114.5, 55.7, 38.6, 14.5. IR (KBr): 3405, 3210, 3137, 3031, 2833, 1587, 1514, 859 cm^{-1}. MS (ESI): *m/z* (100%) = 238 (M + 1). Anal. Calcd for $C_{11}H_{15}N_3OS$ (237.32): C, 55.67; H, 6.37; N, 17.71. Found: C, 55.61; H, 6.32; N, 17.76.

5.2.10. 1-(4-(4-Hydroxyphenyl)butan-2-ylidene)-thiosemicarbazide (**1j**)

M.p. 154–155 °C. $R_f = 0.69$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, DMSO-d_6): δ 8.49 (1H, br s, NH), 7.12 (1H, br s, NH_2), 7.02 (2H, d, $J = 6.3$ Hz,

phH), 6.77 (2H, d, $J = 6.3$ Hz, phH), 6.22 (1H, br s, NH_2), 2.80 (2H, t, $J = 7.2$ Hz, CH_2), 2.57 (2H, t, $J = 7.2$ Hz, CH_2), 1.89 (3H, s, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.1, 159.3, 148.9, 129.1, 128.8, 115.7, 32.5, 29.3, 19.8. IR (KBr): 3609, 3366, 3265, 3187, 1610, 1513, 826 cm^{-1}. MS (ESI): *m/z* (100%) = 236 (M − 1). Anal. Calcd for $C_{11}H_{15}N_3OS$ (237.32): C, 55.67; H, 6.37; N, 17.71. Found: C, 55.73; H, 6.35; N, 17.79.

*5.2.11. 1-Cyclopentylidenethiosemicarbazide (**2a**)*

M.p. 152–154 °C. $R_f = 0.65$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, DMSO-d_6): δ 8.45 (1H, br s, NH), 7.16 (1H, br s, NH_2), 6.44 (1H, br s, NH_2), 2.39 (2H, t, $J = 6.6$ Hz, CH_2), 2.31 (2H, t, $J = 6.6$ Hz, CH_2), 1.89–1.91 (2H, m, CH_2), 1.78–1.80 (2H, m, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6): δ 178.5, 164.0, 33.7, 28.5, 25.3. IR (KBr): 3383, 3261, 3138, 2957, 1661 cm^{-1}. MS (ESI): *m/z* (100%) = 158 (M + 1). Anal. Calcd for $C_6H_{11}N_3S$ (157.24): C, 45.83; H, 7.05; N, 26.72. Found: C, 45.95; H, 7.09; N, 26.85.

*5.2.12. 1-Cyclohexylidenethiosemicarbazide (**2b**)*

M.p. 154–155 °C. $R_f = 0.65$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, DMSO-d_6): δ 8.82 (1H, br s, NH), 7.26 (1H, br s, NH_2), 6.46 (1H, br s, NH_2), 2.25–2.33 (4H, m, $2CH_2$), 1.63–1.71 (6H, m, $3CH_2$). ^{13}C NMR (75 MHz, DMSO-d_6): δ 178.8, 157.5, 27.4, 26.3, 25.8. IR (KBr): 3379, 3217, 3144, 2939, 2858, 1585 cm^{-1}. MS (ESI): *m/z* (100%) = 172 (M + 1). Anal. Calcd for $C_7H_{13}N_3S$ (171.26): C, 49.09; H, 7.65; N, 24.54. Found: C, 49.06; H, 7.63; N, 24.75.

*5.2.13. 1-(3-Oxocyclohexylidene)thiosemicarbazide (**3**)*

M.p. 175–176 °C. $R_f = 0.66$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, DMSO-d_6): δ 11.85 (1H, br s, NH), 9.20 (1H, br s, NH_2), 7.38 (1H, br s, NH_2), 3.42 (2H, s, CH_2), 2.21–2.41 (4H, m, $2CH_2$), 1.71–1.72 (2H, m, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6): δ 208.7, 178.6, 159.2, 46.8, 44.7, 22.5, 19.2. IR (KBr): 3416, 3275, 3161, 1716, 1603 cm^{-1}. MS (ESI): *m/z* (100%) = 186 (M + 1).

*5.2.14. 1-(2,5-Dimethyl-1H-pyrrol-1-yl)thiourea (**4**)*

M.p. 217–218 °C. $R_f = 0.75$ (ethyl acetate/petroleum ether (bp 60–90 °C) = 2:1, v/v). ^1H NMR (300 MHz, DMSO-d_6): δ 10.19 (1H, br s, NH), 8.18 (1H, br s, NH_2), 6.54 (1H, br s, NH_2), 5.68 (2H, s, 2CH), 1.99 (6H, s, $2CH_3$). ^{13}C NMR (75 MHz, DMSO-d_6): δ 183.15, 127.64, 104.71, 11.73. IR (KBr): 3385, 3231, 3146, 1956, 1616 cm^{-1}. MS (ESI): *m/z* (100%) = 170 (M + 1). Anal. Calcd for $C_7H_{11}N_3S$ (169.25): C, 49.68; H, 6.55; N, 24.83. Found: C, 49.59; H, 6.51; N, 25.21.

6. Tyrosinase assay

Tyrosinase inhibition assays were performed according to the method developed by Hearing [19] with slight modification. Briefly, all the synthesized compounds were screened for the *o*-diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All the active inhibitors from the preliminary screening were subjected to IC_{50} studies. All the synthesized compounds were dissolved in DMSO to a concentration of 2.0%. Phosphate buffer pH 6.8 was used to dilute the DMSO stock solution of test compound. Thirty units of mushroom tyrosinase (0.2 μg/mL) were first pre-incubated with the compounds, in 50 mM phosphate buffer (pH 6.8), for 10 min at 25 °C. Then L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm for formation of the DOPA chrome for 1 min. Dose–response curves were obtained by performing assays in the presence of increasing concentrations of inhibitors (at least eight concentrations). IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose–response curves. The percent of inhibition of tyrosinase reaction was calculated as following:

$$\text{Inhibition rate}(\%) = [(B - S)/B] \times 100$$

Here, the B and S are the absorbances for the blank and samples, respectively. 4-Methoxycinnamic acid and arbutin were used as reference standard inhibitors for comparison. All the studies were carried out at least in triplicate and the results here represent mean ± SEM (standard error of the mean).

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