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Design and synthesis of thiourea derivatives with sulfur-containing heterocyclic scaffolds as potential tyrosinase inhibitors

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ABSTRACT

Tyrosinase is a key enzyme during the production of melanins in plants and animals. A class of novel *N*-aryl-*N*′-substituted phenylthiourea derivatives (**3a**–**i**, **6a**–**k**) were designed, synthesized and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were evaluated. The results showed some 4,5,6,7-tetrahydro-2-[[(phenylamino)thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid derivatives (**3a**–**i**) exhibited moderate inhibitory potency on diphenolase activity of tyrosinase. When the scaffold of 4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid was replaced with 2-(1,3,4-thiadiazol-2-yl)thio acetic acid, the inhibitory activity of compounds (**6a**–**k**) against tyrosinase was improved obviously; especially, the inhibitory activity of compound **6h** (IC_{50} = 6.13 μM) is significantly higher than kojic acid (IC_{50} = 33.3 μM). Moreover, the analysis on inhibition mechanism revealed that compound **6h** might plays the role as a noncompetitive inhibitor.

1. Introduction

Tyrosinase (polyphenol oxidase, EC 1.14.18.1),[1] a multifunctional copper-containing polyphenol oxidative enzyme widely distributed in plants, microorganisms, fungi and animals, catalyzes two types of oxidative reactions: hydroxylation of monophenols to o-diphenols, which are oxidized to the corresponding *o*-quinones.[2] Quinones can polymerize non-enzymatically to melanin as the most important natural biopolymer responsible of pigmentation and the color and patterns of mammalian skin. It had also been reported that the tyrosinase might be relevant to melanoma.[3] In agriculture areas, tyrosinase is responsible for the undesired enzymatic browning of fruits and vegetables that take place during senescence or damage at the time of post-harvest handling. The browning pigments lead to organoleptic and nutritional modifications, thus depreciating the quality of the food product.[4]

Tyrosinase inhibitors were widely used in agriculture and food industry, as well as in medicinal and cosmetic products due to preventing oxidization and decreasing the excessive accumulation of pigmentation.[5] Many tyrosinase inhibitors are widely used in cosmetic products for whitening and depigmentation after sunburn. Moreover, tyrosinase inhibitors could be used in the control of the molting process of insect.[6] With the increasing concern on health issues, tyrosinase was also used as a main target in research of skin-whitening products. As a commonly used inhibitor, kojic acid has been banned as cosmetic ingredient in many countries because of its serious side effects.[6] However, as a phenolic compound, kojic acid (Scheme 1, a) is unstable under aerobic condition, especially in solutions. Hence, more efforts should be made in the development of effective and stable novel tyrosinase inhibitors with lower side effects.

A given structure of two copper ions in the active center of tyrosinase and a lipophilic long-narrow gorge near to the active center provided ideal models to develop original tyrosinase inhibitor. Over the past decades, a broad spectrum of natural and synthetic compounds, such as Resveratrol (Scheme 1, b),[7] chalcone derivatives (Scheme 1, c),[8] kojic acid and derivatives,[9] thiocarbonyl-containing benzylidene derivatives (Scheme 1, d),[10] thiosemicarbazone analogues (Scheme 1, e)[11] and phenylthiourea derivatives (Scheme 1, f),[12] have been described to inhibit tyrosinase.

Most of those compounds have inherent function in chelating metal ions which plays a critical role in the inhibition of tyrosinase. As be well known, sulfur-containing moieties, such as thiocarbonyl in thioureas or thiosemicarbazones, sulfur atoms on heterocycles, can chelate transition metal ions.[11f,g,i] In the present work, initially, we designed and synthesized a serial of novel *N*-aryl-*N*′-substituted phenyl thiourea derivatives (Scheme 1, **3a**–**i**) in order to discover efficient tyrosinase inhibitors. Thiophene scaffold was introduced into the structure because sulphur-containing heterocycles were

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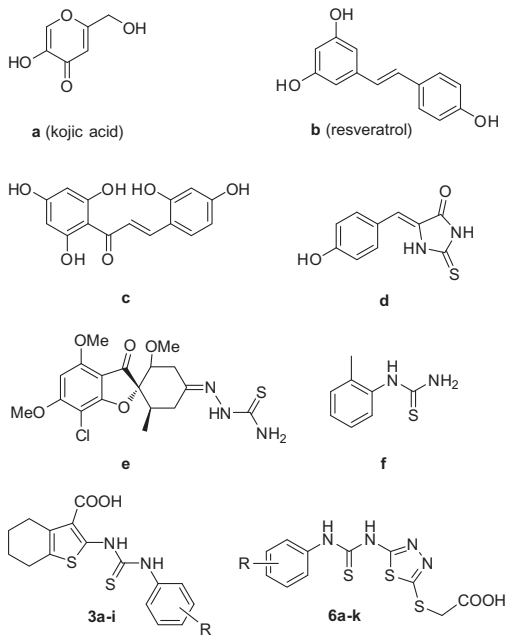
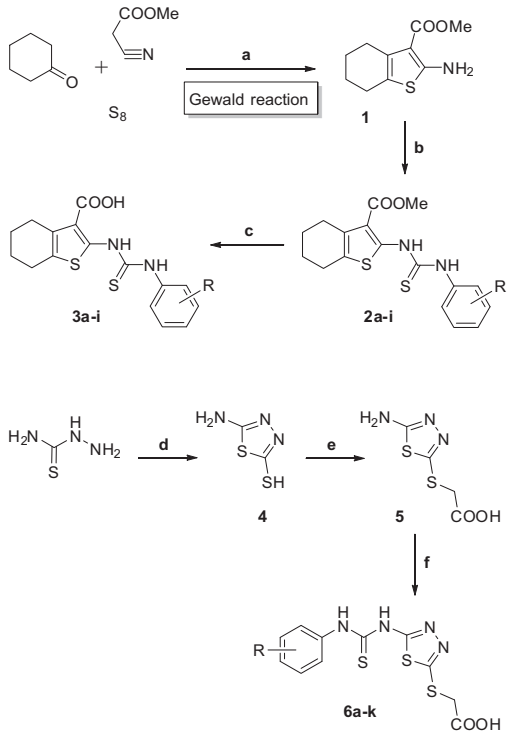


Scheme 1.

expected to be effective in chelating with copper ion. At the beginning of this work, 4,5,6,7-tetrahydro-2-[[(substitutedphenylamino) thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylate derivatives (**2a–i**) were synthesized by the designed route (Scheme 2). Unfortunately, it was very difficult to dissolve these compounds in aqueous buffer solution containing enzyme due to their poor solubility so the activity of compounds **2a–i** could not be evaluated.



Scheme 2. Synthesis of compounds **3a–i**, **6a–k**. Reagents and conditions: (a) morpholine, EtOH, reflux; (b) aryl isothiocyanates, ethanol, reflux; (c) ethanol, 20% NaOH, reflux; (d) CS_2, NaOH, ethanol, reflux; (e) $ClCH_2COOH$, NaOH, H_2O, 60 °C; (f) aryl isothiocyanates, Et_3N, DMF, CH_3CN, 50 °C.

Considering carboxylic acids have relatively better solubility in aqueous phase, moreover, some carboxylic acids themselves were reported showed inhibitory effect on tyrosinase.[13] Hence, carboxylates **2** were then transformed to corresponding carboxylic acids **3** by hydrolysis in alkaline solution.

To our delight, some of these compounds exhibit inhibitory effect on tyrosinase, although the activities were lower than kojic acid. Encouraged by these results, we decided to extend the structural diversity by the replacement of the thiophene scaffold of **3** with 1,3,4-thiadiazol containing thioether-type side chain. Subsequently, *N*-phenyl-*N′*-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl] thiourea derivatives (**6a–k**) were synthesized as represented in Scheme 2. In comparison with **3**, the inhibitory activities of compounds **6** against tyrosinase were distinctly enhanced. To explore the inhibition mechanism of these compounds, the inhibitory kinetics of compound **6h** was also studied in detail.

2. Experimental section

2.1. General

Melting points were measured on BUCHI B-450 melting point apparatus. ^1H and ^{13}C NMR spectra were recorded on a Bruker AM-400 (400 MHz) spectrometer. Mass spectral analyses were performed on an Agilent mass spectrometer under electron spray ionization. Mushroom tyrosinase (EC 1.14.18.1) was purchased from Worthington Biochemical Corporation. All other reagents were commercial products with analytical grade purity and used as received.

2.2. Chemistry

2.2.1. Synthesis of compounds 1, 2a–i and 3a–i

Compound **1** was prepared through standard Gewald reaction.[14] 4.4 g (0.05 mol) of morpholine was added dropwise into a stirred solution of cyclohexanone (4.91 g, 0.05 mol), methyl cyanoacetate (4.95 g, 0.05 mol) and sulfur (1.92 g, 0.06 mol) in 35 mL ethanol. On completion, the mixture was refluxed for further 3 h. After cooling to room temperature, the precipitate was separated by filtration and recrystallized from ethanol to give **1** as pale yellow powders (8.6 g, 82% yield), mp 128.2–129.4 °C (lit.[15] 128–130 °C), MS (GC–MS): *m/z*, 211 $[M+H]^+$, 179, 151, 125, 91, 77, 65, 53.

To the stirred solution of compound **1** (0.22 g, 2 mmol) in 3 mL absolute ethanol, 2 mmol of aryl isothiocyanate was added and refluxed for 13–16 h under the atmosphere of nitrogen. The solvent was removed by rotary evaporation and the crude product was washed with cold ethanol, dried, and recrystallized from ethanol to afford compounds **2a–i**.

10 mL of aqueous NaOH solution (20% wt) was added into the stirred solution of compounds **2a–i** in ethanol (5 mL). The resulting mixture was refluxed for 2 h. Subsequently, the solvent was removed by rotary evaporation, and the residue was added 20 mL of water followed by extracted with dichloromethane (10 mL). The water layer was neutralized with diluted hydrochloric acid to afford crude product as a precipitate which was then collected by filtration. Recrystallization from ethanol gave purified compounds **3a–i**.

2.2.1.1. 4,5,6,7-Tetrahydro-2-[[(phenylamino)thioxomethyl] amino]-benzo[*b*]thiophene-3-carboxylic acid (3a). Yield 86%, white solid, mp 241.3–241.7 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 7.48–7.44 (m, 2H, Ar-H), 7.40–7.37 (m, 1H, Ar-H), 7.21 (d, J = 7.6 Hz, 2H, Ar-H), 2.73–2.68 (m, 4H, 2 × CH_2), 1.79–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 179.72, 162.12, 154.50, 144.53, 136.37, 134.23, 134.13, 133.54, 133.22,

121.35, 30.05, 29.19, 27.70, 26.78 ppm; MS (LC–MS): m/z 315.1 $[M-17]^+$. Anal. Calcd for $C_{16}H_{16}N_2O_2S_2$: C, 57.81; H, 4.85; N, 8.43. Found: C, 57.89; H, 4.92; N, 8.38.

2.2.1.2. 4,5,6,7-Tetrahydro-2-[[(4-methylphenylamino)thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid (3b). Yield 76%, white solid, mp 345.5–345.8 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.63 (s, 1H, COOH), 7.25 (d, J = 6.8 Hz, 2H, Ar-H), 7.07 (d, J = 7.2 Hz, 2H, Ar-H), 2.72–2.68 (m, 4H, 2 × CH_2), 2.36 (s, 3H, CH_3), 1.77–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 175.08, 157.43, 149.67, 137.75, 137.17, 131.62, 129.92, 129.15, 128.75, 116.57, 25.31, 24.43, 22.94, 22.02, 21.25 ppm; MS (LC–MS): m/z 329.1 $[M-OH]^+$. Anal. Calcd for $C_{17}H_{18}N_2O_2S_2$: C, 58.93; H, 5.24; N, 8.09. Found: C, 59.02; H, 5.31; N, 8.02.

2.2.1.3. 4,5,6,7-Tetrahydro-2-[[(4-fluorophenylamino)thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid (3c). Yield 86%, white solid, mp 342.6–343.1 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.69 (s, 1H, COOH), 7.28 (d, J = 6.8 Hz, 4H, Ar-H), 2.73–2.68 (m, 4H, 2 × CH_2), 1.79–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 175.05, 157.42, 149.75, 136.00, 135.97, 131.69, 131.61, 128.85, 116.59, 116.36, 116.13, 25.29, 24.43, 22.94, 22.02 ppm; MS (LC–MS): m/z 333.1 $[M-17]^+$. Anal. Calcd for $C_{16}H_{15}FN_2O_2S_2$: C, 54.84; H, 4.31; N, 7.99. Found: C, 54.92; H, 4.38; N, 7.93.

2.2.1.4. 4,5,6,7-Tetrahydro-2-[[(4-chlorophenylamino)thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid (3d). Yield 81%, white solid, mp 352.6–352.9 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.52 (d, J = 7.6 Hz, 2H, Ar-H), 7.28 (d, J = 7.2 Hz, 2H, Ar-H), 2.73–2.68 (m, 4H, 2 × CH_2), 1.79–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 174.24, 156.72, 149.24, 138.15, 132.52, 131.05, 130.97, 128.91, 128.32, 116.02, 24.72, 23.86, 22.37, 21.44 ppm; MS (LC–MS): m/z 349.0 $[M-17]^+$. Anal. Calcd for $C_{16}H_{15}ClN_2O_2S_2$: C, 52.38; H, 4.12; N, 7.64. Found: C, 52.44; H, 4.21; N, 7.58.

2.2.1.5. 4,5,6,7-Tetrahydro-2-[[(4-bromophenylamino)thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid (3e). Yield 75%, white solid, mp 317.1–317.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.65 (d, J = 8.8 Hz, 2H, Ar-H), 7.20 (d, J = 8.4 Hz, 2H, Ar-H), 2.72–2.67 (m, 4H, 2 × CH_2), 1.78–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 174.16, 156.70, 149.29, 138.63, 131.90, 131.34, 131.05, 128.37, 121.10, 116.03, 24.73, 23.88, 22.39, 21.46 ppm; MS (LC–MS): m/z 395.0 $[M-15]^+$. Anal. Calcd for $C_{16}H_{15}ClN_2O_2S_2$: C, 46.72; H, 3.68; N, 6.81. Found: C, 46.78; H, 3.76; N, 6.74.

2.2.1.6. 4,5,6,7-Tetrahydro-2-[[(2-methylphenylamino)thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid (3f). Yield 79%, white solid, mp 325.8–326.1 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.71 (s, 1H, COOH), 7.31–7.26 (m, 3H, Ar-H), 7.12 (d, J = 6.8 Hz, 1H, Ar-H), 2.73–2.68 (m, 4H, 2 × CH_2), 2.02 (s, 3H, CH_3), 1.78–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 173.63, 156.33, 149.42, 138.15, 135.13, 131.06, 130.38, 128.71, 128.43, 128.21, 126.72, 115.81, 24.72, 23.88, 22.35, 21.44, 16.87 ppm; MS (LC–MS): m/z 329.1 $[M-17]^+$. Anal. Calcd for $C_{17}H_{18}N_2O_2S_2$: C, 58.93; H, 5.24; N, 8.09. Found: C, 59.01; H, 5.32; N, 8.04.

2.2.1.7. 4,5,6,7-Tetrahydro-2-[[(3-fluorophenylamino) thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid (3g). Yield 76%, white solid, mp 321.2–321.3 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.73 (s, 1H, COOH), 7.51–7.45 (m, 2H, Ar-H), 7.41 (d, J = 7.2 Hz, 1H, Ar-H), 7.23 (d, J = 6.8 Hz, 1H, Ar-H), 2.73–2.68 (m, 4H, 2 × CH_2), 1.78–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 174.15, 156.69, 149.29, 140.57, 132.86, 131.06, 130.39, 129.18, 128.35, 128.09, 128.06, 116.04, 24.72, 23.87, 22.38, 21.46 ppm; MS (LC–MS): m/z 349.0 $[M-H]^+$. Anal. Calcd for $C_{16}H_{15}FN_2O_2S_2$: C, 54.84; H, 4.31; N, 7.99. Found: C, 54.92; H, 4.36; N, 7.94.

2.2.1.8. 4,5,6,7-Tetrahydro-2-[[(3-chlorophenylamino) thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid (3h). Yield 81%, white solid, mp 326.2–326.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.73 (s, 1H, COOH), 7.51–7.45 (m, 2H, Ar-H), 7.41 (s, 1H, Ar-H), 7.23 (d, J = 7.2 Hz, 1H, Ar-H), 2.72–2.68 (m, 4H, 2 × CH_2), 1.78–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 174.15, 156.70, 149.29, 140.58, 132.87, 131.05, 130.40, 129.18, 128.37, 128.09, 128.06, 116.05, 24.72, 23.88, 22.39, 21.46 ppm; MS (LC–MS): m/z 349.0 $[M-17]^+$. Anal. Calcd for $C_{16}H_{15}ClN_2O_2S_2$: C, 52.38; H, 4.12; N, 7.64. Found: C, 52.46; H, 4.19; N, 7.56.

2.2.1.9. 4,5,6,7-Tetrahydro-2-[[(3,4-difluorohenylamino) thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acid (3i). Yield 91%, white solid, mp 343.4–343.8 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.74 (s, 1H, COOH), 7.57–7.48 (m, 2H, Ar-H), 7.16–7.14 (m, 1H, Ar-H), 2.73–2.68 (m, 4H, 2 × CH_2), 1.77–1.71 (m, 4H, 2 × CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 174.30, 156.71, 149.27, 135.88, 131.06, 128.40, 126.56, 126.54, 118.97, 118.78, 117.52, 117.34, 116.02, 24.72, 23.87, 22.38, 21.45 ppm; MS (LC–MS): m/z 351.0 $[M-17]^+$. Anal. Calcd for $C_{16}H_{14}F_2N_2O_2S_2$: C, 52.16; H, 3.83; N, 7.60. Found: C, 52.24; H, 3.91; N, 7.54.

2.2.2. Synthesis of 4, 5 and 6a–k

Carbon disulfide (7.6 g, 0.1 mol) was added dropwise into an ice-cooled solution of thiosemicarbazide (4.55 g, 0.05 mol) and NaOH (2.4 g, 0.06 mol) in ethanol (20 mL). On completion, the mixture was stirred for 0.5 h at room temperature, and then refluxed for further 5 h. After cooling, 90 mL of water was added and the insoluble solid was filtered off. The filtrate was neutralized with hydrochloric acid and the precipitates were collected by filtration. Recrystallization of crude product from aqueous ethanol provided pure product as pale yellow crystals in 76% yield. Mp 232.1–232.8 °C (lit.[16] 230–232 °C), MS (LC–MS): m/z 133.1 $[M^+]$, 117.1, 105.1, 91.1, 74.1, 56.0.

The mixture of 2-amino-5-mercapto-1,3,4-thiadiazole **4** (5.33 g, 0.04 mol), chloroacetic acid (3.78 g, 0.04 mol) and NaOH (3.2 g, 0.08 mol) in 50 mL water was heated at 60 °C for 4 h. After cooling to room temperature, the solution was neutralized with hydrochloric acid. The precipitate was filtered and then recrystallized from aqueous ethanol (5:1) to obtain purified **5** as pale yellow crystalline solid. Yield 75%, mp 238.4–239.1 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.82 (s, 1H, COOH), 7.28 (s, 2H, NH_2), 3.90 (s, 2H, CH_2) ppm; MS (EI): m/z 191.1 $[M^+]$ (28), 147.0 (44), 133.1 (100), 114.0 (38), 74.1 (19).

0.95 g (0.005 mol) of 2-[(5-amino-1,3,4-thiadiazol-2-yl)thio] acetic acid **5** and Et_3N (1.01 g, 0.01 mol) was dissolved in 5 mL DMF and 20 mL CH_3CN. Subsequently, aryl isothiocyanate (0.005 mol) was added slowly into above-mentioned solution under the atmosphere of nitrogen. The resulting mixture was heated for 5 h at 50 °C and CH_3CN was removed by rotary evaporation. After cooling, 20 mL of water was added and the insoluble impurities were isolated by filtration. The filtrate was neutralized with hydrochloric acid. The crude product thus precipitated was filtered and recrystallized from ethanol to afford desired compounds **6a–k**.

2.2.2.1. *N*-Phenyl-*N*′-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl]thiourea (6a). Yield 59%, yellow solid, mp 211.7–212.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.49 (s, 1H, COOH), 9.79 (s, 1H, NH),

9.06 (s, 1H, NH), 7.66 (d, J = 7.6 Hz, 2H, Ar-H), 7.34 (t, J = 8.0 Hz, 2H, Ar-H), 7.13 (t, J = 7.2 Hz, 1H, Ar-H), 4.07 (s, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ: 182.64, 169.32, 153.70, 139.34, 128.94, 128.45, 124.40, 122.74, 118.92, 34.88 ppm; HRMS (ESI-MS) calcd. for $C_{11}H_9N_4O_2S_3$ $[M-H]^+$:324.9888, Found: 324.9891.

2.2.2.2. *N*-(4-Methylphenyl)-*N′*-[5-(carboxymethyl thio)-1,3,4-thiadiazol-2-yl]thiourea (6b). Yield 52%, brownish solid, mp 238.1–238.3 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.40 (s, 1H, COOH), 9.98 (s, 1H, NH), 9.03 (s, 1H, NH), 7.50 (d, J = 7.8 Hz, 2H, Ar-H), 7.14 (d, J = 8.0 Hz, 2H, Ar-H), 4.08 (s, 2H, CH_2), 2.28 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 181.95, 169.55, 156.77, 136.76, 130.23, 128.91, 123.02, 119.24, 35.98, 20.54 ppm; HRMS (ESI-MS): calcd. for $C_{12}H_{11}N_4O_2S_3$ $[M-H]^+$: 339.0044, found: 339.0042.

2.2.2.3. *N*-(4-Fluorophenyl)-*N′*-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl]thiourea (6c). Yield 42%, yellow solid, mp 247.6–247.8 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.47 (s, 1H, COOH), 9.64 (s, 1H, NH), 9.13 (s, 1H, NH), 7.28–7.15 (m, 4H, Ar-H), 3.89 (s, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 181.10, 169.63, 155.30, 149.53, 137.69, 135.08, 130.31, 128.22, 126.14, 36.09 ppm; HRMS (ESI-MS): calcd. for $C_{11}H_{10}FN_4O_2S_3$ $[M+H]^+$: 344.9950, found: 344.9934.

2.2.2.4. *N*-(4-Chlorophenyl)-*N′*-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl]thiourea (6d). Yield 54%, yellow solid, mp 204.8–205.2 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.12 (s, 1H, COOH), 9.55 (s, 1H, NH), 8.83 (s, 1H, NH), 7.52 (d, J = 7.2 Hz, 2H, Ar-H), 7.02 (d, J = 7.6 Hz, 2H, Ar-H), 4.07 (s, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 181.43, 169.97, 149.86, 138.02, 135.41, 130.64, 128.55, 126.47, 36.41 ppm; HRMS (ESI-MS): calcd. for $C_{11}H_{10}ClN_4O_2S_3$ $[M-H]^+$: 358.9498, found: 358.9496.

2.2.2.5. *N*-(2-Methoxyphenyl)-*N′*-[5-(carboxymethylthio)-1,3,4-thiadiazol-2yl]thiourea (6e). Yield 55%, yellow solid, mp 202.4–202.5 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.81 (s, 1H, COOH), 9.76(s, 1H, NH), 9.56 (s, 1H, NH), 7.91 (d, J = 6.8 Hz, 1H, Ar-H), 6.90–7.18 (m, 3H, Ar-H), 4.04 (s, 2H, CH_2), 3.81 (s, 3H, OCH_3) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 179.54, 169.68, 162.58, 156.87, 149.45, 127.42, 125.07, 120.42, 120.14, 111.61, 55.88, 35.71 ppm; HRMS (ESI-MS): calcd. for $C_{12}H_{13}N_4O_3S_3$ $[M+H]^+$: 357.0150, found: 357.0153.

2.2.2.6. *N*-(3-Fluorophenyl)-*N′*-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl]thiourea (6f). Yield 59%, yellow solid, mp 214.9–215.4 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.62 (s, 1H, COOH), 10.07 (s, 1H, NH), 9.34 (s, 1H, NH), 7.54–7.58 (m, 2H, Ar-H), 7.45–7.46 (m, 1H, Ar-H), 7.36–7.38 (m, 1H, Ar-H), 4.07 (s, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 188.25, 169.29, 163.27, 153.74, 141.13, 140.20, 130.55, 129.98, 119.23, 114.96, 34.74 ppm; HRMS (ESI-MS): calcd. for $C_{11}H_{10}FN_4O_2S_3$ $[M+H]^+$: 344.9950, found: 344.9934.

2.2.2.7. *N*-(3-Chlorophenyl)-*N′*-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl]thiourea (6g). Yield 52%, yellow solid, mp 175.4–175.6 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.62 (s, 1H, COOH), 10.07 (s, 1H, NH), 9.32 (s, 1H, NH), 7.81–7.86 (m, 1H, Ar-H), 7.35–7.46 (m, 2H, Ar-H), 7.15–7.18 (m, 1H, Ar-H), 4.09 (s, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 183.71, 169.53, 153.99, 150.06, 141.05, 132.98, 130.71, 123.59, 118.91, 115.50, 36.30 ppm; HRMS (ESI-MS): calcd. for $C_{11}H_{10}ClN_4O_2S_3$ $[M-H]^+$: 358.9498, found: 358.9496.

2.2.2.8. *N*-[3-(Trifluoromethyl)phenyl]-*N′*-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl] thiourea (6h). Yield 61%, yellow solid, mp 229.1–229.3 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.51 (s, 1H, COOH), 9.16 (s, 1H, NH), 7.56 (s, 1H, Ar-H), 7.47 (d, J = 8.0 Hz, 1H, Ar-H), 7.35 (d, J = 8.0 Hz, 1H, Ar-H), 6.97–7.01 (m, 1H, Ar-H), 3.94 (s, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 183.20, 169.77, 149.72, 139.81, 138.85, 128.58, 127.76, 126.46, 125.15, 125.06, 122.35, 120.68, 119.65, 115.26, 36.20 ppm; HRMS (ESI-MS): calcd. for $C_{12}H_8F_3N_4O_2S_3$ $[M-H]^+$:392.9767, found: 392.9761.

2.2.2.9. *N*-(2,4-Difluorophenyl)-*N′*-[5-(carboxymethyl-thio)-1,3,4-thiadiazol-2-yl]thiourea (6i). Yield 56%, yellow solid, mp 215.1–215.8 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.19 (s, 1H, COOH), 8.96 (s, 1H, NH), 7.59 (d, J = 6.8 Hz, 1H, Ar-H), 7.28–7.32 (m, 1H, Ar-H), 7.08 (s, 1H, Ar-H), 4.14 (s, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 190.23, 169.75, 167.13, 159.07, 152.99, 138.15, 125.66, 115.08, 111.56, 104.39, 35.73 ppm; HRMS (ESI-MS): calcd. for $C_{11}H_9F_2N_4O_2S_3$ $[M+H]^+$:362.9856, found: 362.9836.

2.2.2.10. *N*-(3,4-Difluorophenyl)-*N′*-[5-(carboxymethyl-thio)-1,3,4-thiadiazol-2-yl]thiourea (6j). Yield 48%, yellow solid, mp 248.4–249.1 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.60 (s, 1H, COOH), 9.97 (s, 1H, NH), 9.33 (s, 1H, NH), 7.36 (s, 1H, Ar-H), 7.12–7.15 (m, 1H, Ar-H), 6.92 (d, J = 8.4 Hz, 1H, Ar-H), 4.02 (s, 2H, CH_2) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 188.76, 166.71, 150.52, 148.09, 144.85, 135.81, 124.62, 118.01, 116.15, 108.95, 36.41 ppm; HRMS(ESI-MS): calcd. for $C_{11}H_9F_2N_4O_2S_3$ $[M+H]^+$: 362.9856, found: 362.9836.

2.2.2.11. *N*-(3-Chloro-4-fluorophenyl)-*N′*-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl] thiourea (6k). Yield 55%, yellow solid, mp 240.2–240.4 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.45 (s, 1H, COOH), 9.55 (s, 1H, NH), 8.83 (s, 1H, NH), 7.53 (s, 1H, Ar-H), 6.87 (s, 1H, Ar-H), 6.53 (s, 1H, Ar-H), 4.07 (s, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 182.64, 171.54, 163.34, 136.84, 131.45, 128.40, 122.74, 118.92, 116.15, 109.58, 36.34 ppm; HRMS (ESI-MS): calcd. for $C_{11}H_9ClFN_4O_2S_3$ $[M+H]^+$: 378.9560, found: 378.9575.

2.3. Tyrosinase activity assay

The inhibitory activity of test compounds against tyrosinase was investigated using previously described procedure[17] with slight modifications. Briefly, the compounds **3a**–**i** and **6a**–**k** were assessed for the diphenolase inhibitory activity against tyrosinase using *L*-DOPA as substrate. Stock solutions of the test compounds were prepared in DMSO; further dilutions were done in DMSO to yield various concentrations using phosphate buffer (pH = 6.8). The 200 μL reaction system included 50 μL diluted solution of test compound, 50 μL phosphate buffer, 50 μL *L*-DOPA and 50 μL tyrosinase solution. The enzyme reaction was monitored by measuring the change in absorbance at 475 nm for 5 min at 30 °C. The measurement was completed in triplicate for each concentration and averaged before further calculation. The phosphate buffer containing 2.0% DMSO instead of the diluted solution was used as the negative control. The kojic acid was used as a positive control.

The determination of inhibition kinetics was performed by modification of the above-mentioned method: for each of four different inhibitor concentrations (0, 3.67, 6.35, and 12.70 μM, respectively). L-DOPA concentration was varied (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0 mM). The inhibition type on the enzyme was assayed by Lineweaver–Burk plots, and the inhibition constant was determined by the second plots of the apparent K_m/V_m (slope of Lineweaver–Burk double reciprocal lines) versus the concentration of compound **6h**.

3. Results and discussion

3.1. Synthesis

As shown in Scheme 2, 2-amino-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carboxylate (**1**) was synthesized by Gewald reaction. Compound **1** reacted with aryl isothiocyanates under nitrogen atmosphere to afford compounds **2a**–**i**, hydrolysis of **2a**–**i** to afford compounds **3a**–**i** in good yields. The structures of the products **3a**–**i** were identified by their ^{1}H NMR, ^{13}C NMR spectrum and mass spectrum data. Their ^{1}H NMR spectrum showed a broad signal at δ 13.7 (COOH).

Aryl isothiocyanates hydrolyzed in the presence of water and led to complex byproducts. Hence, the solvents employed must be pre-dried, and the reaction should be carried out under the atmosphere of nitrogen.

The reaction of 2-amino-5-mercapto-1,3,4-thiadiazole (**4**) and chloroacetic acid could be carried out in aqueous phase to afford 2-[(5-amino-1,3,4-thiadiazol-2-yl)thio] acetic acid (**5**). The hydrolysis of chloroacetic acid became a serious problem at elevated temperatures; thus the reaction temperature should be controlled at about 60 °C to avoid the side reaction. The compound (**5**) reacted with aryl isothiocyanates at 50 °C for 5 h to afford compounds **6a**–**k** in moderate yield. The structures of the products **6a**–**k** were supported by their ^{1}H NMR, ^{13}C NMR spectrum and mass data.

The ^{1}H NMR spectrum of compound **5** shows a signal at δ 7.28 (s, 2H, NH_2). The ^{1}H NMR spectrum of compound **6** showed three broad signals at δ 11 ~ 9 (COOH, NH, NH). Their structures were confirmed by their mass spectra which showed peaks corresponding to their molecular ion.

3.2. Biological activity

3.2.1. Inhibitory effects on the diphenolase activity of tyrosinase

The inhibition of as-synthesized 4,5,6,7-tetrahydro-2-[[(substitutedphenylamino)thioxomethyl]amino]-benzo[*b*]thiophene-3-carboxylic acids **3a**–**i** on the tyrosinase was investigated and compared with kojic acid at 75 μM. As shown in Table 1, part of these compounds exhibited inhibitory activity on tyrosinase. However, their activities were lower than kojic acid.

We speculated that the carboxyl group neighbor thiourea pharmacophore might retard the binding of inhibitor to the active site of tyrosinase. Considering thiadiazole, oxadiazole and triazole rings monodentate binding to the active site dicopper center of tyrosinase,[11f] dihydro-asparagusic acid having inhibitory effect on tyrosinase,[18] we designed a serial of compounds **6a**–**k** with the scafford 2-((1,3,4-thiadiazol-2-yl)thio)acetic acid connected to thiourea pharmacophore which were prospected to be efficient in chelating with the dicopper active center. These compounds could be prepared readily by the reaction between 2-[(5-amino-1,3,4-thiadiazol-2-yl)thio]acetic acid and isothiocyanates.

Inhibitory activity against tyrosinase (diphenolase) of compounds **6a**–**k** was investigated. As shown in Table 2, all of *N*-phenyl-*N*′-[5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl]thiourea derivatives displayed potent tyrosinase inhibitory activities. Especially, the obtained compounds **6c** (IC_{50} = 20.9 μM), **6h** (IC_{50} = 6.13 μM), **6i** (IC_{50} = 20.1 μM) and **6j** (IC_{50} = 20.5 μM) exhibited more potent tyrosinase inhibitory activities than the reference inhibitor kojic acid (IC_{50} = 33.3 μM).

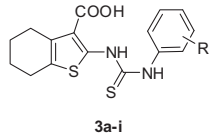
These results showed that the introduction of 2-[(5-amino-1,3,4-thiadiazol-2-yl)thio] acetic acid substructure was crucial for presenting the inhibitory effect on tyrosinase.The data in Table 2 shows that compounds including fluorine atoms at phenyl ring exhibited more potent tyrosinase inhibitory activities.

3.2.2. Inhibitory mechanism of compound 6h on mushroom tyrosinase

Finally we determined the inhibitory type of selected compound **6h** from Lineweaver–Burk double reciprocal plots. In the presence of **6h**, the kinetics of the enzyme was shown in Figure 1a. The results displayed that the plots of 1/*V* versus 1/[*S*] gave four straight lines with different slopes and intersected on the horizontal axis. When the concentration of compound **6h** was elevated, the V_{max} value decreased, whereas the K_m value was unchanged, suggesting compound **6h** is regarded as a noncompetitive inhibitor of mushroom tyrosinase. The inhibition constant (K_I = 8.13 μM) was obtained by plotting the slope values versus the concentration of the compound **6h**, as shown in Figure 1b.

Many thiourea derivatives were known to be competitive inhibitors of tyrosinase due to their chelating ability to copper ions at the active sites. Interestingly, the Lineweaver–Burk analysis revealed a non-competitive behavior of compound **6h**. Although the title compounds were designed initially as copper-chelating competitive inhibitors, the experimental results reveal that they might act through allosteric effects.

Table 1
Structures and inhibitory activity against tyrosinase (diphenolase) of compounds **3a**–**i** at 75 μM

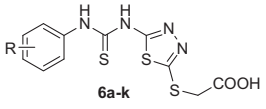


3a-i

Compounds No.	R	% inhibition
3a	H	53.3 ± 0.5[a]
3b	4-CH_3	NI[b]
3c	4-F	44.3 ± 1.5
3d	4-Cl	NI
3e	4-Br	31.7 ± 1.3
3f	2-CH_3	42.1 ± 0.5
3g	3-F	44.8 ± 0.3
3h	3-Cl	NI
3i	3,4-Di-F	44.7 ± 0.3
Positive control	Kojic acid	88.5 ± 0.3

[a] SEM: standard error of the mean.
[b] NI: no inhibitory activity.

Table 2
Structures and inhibitory activity against tyrosinase (diphenolase) of compounds **6a**–**k**



6a-k

Compounds No.	R	IC_{50} (μM)
6a	H	27.7 ± 1.2
6b	4-CH_3	54.4 ± 0.8
6c	4-F	20.9 ± 1.2
6d	4-Cl	26.3 ± 0.5
6e	2-OCH_3	27.1 ± 0.6
6f	3-F	31.3 ± 0.8
6g	3-Cl	59.7 ± 0.5
6h	3-CF_3	6.13 ± 0.3
6i	2,4-Di-F	20.1 ± 1.2
6j	3,4-Di-F	20.5 ± 0.8
6k	3-Cl-4-F	33.9 ± 1.2
Positive control	Kojic acid	33.3 ± 1.1

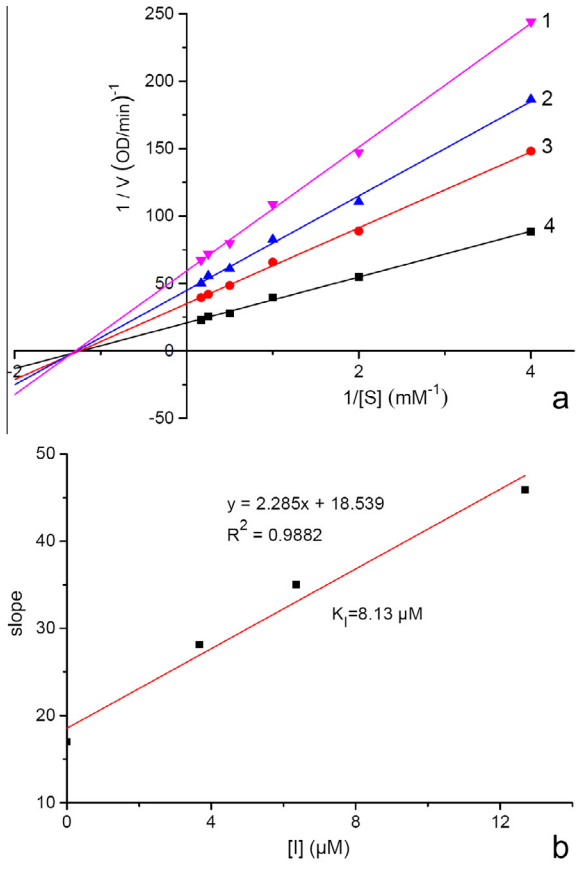


Figure 1. Determination of the inhibitory type and inhibition constant of compound **6h**. The concentrations of 6 h for curves 1–4 were 12.70, 6.35, 3.67 and 0 μM, respectively. (a) Lineweaver–Burk plots for inhibition of compound **6h** on the activity of tyrosinase. (b) The plot of slope versus the concentration of compound **6h** for determining the inhibition constant K_I.

4. Conclusions

In summary, on the basis of the initial thiofuran-type lead compound identified from screening a small focused library, we have discovered 5-(carboxymethylthio)-1,3,4-thiadiazol-2-yl thiourea as a novel scaffold for tyrosinase inhibitors. The newly synthesized compounds exhibit a remarkable inhibitory effect on the diphenolase activity of tyrosinase, therefore are promising for their uses as tyrosinase inhibitors. The results showed that several compounds, especially the compound **6h**, had higher inhibitory activity against tyrosinase in comparison with the widely used tyrosinase inhibitor kojic acid. The inhibition mechanism studies revealed that the compounds prepared in this study exhibited inhibitory effect on tyrosinase by acting as the noncompetitive inhibitor.

Thus, given the observed inhibitory effects on tyrosinase, we believe that sulfur-containing heterocyclic thiourea derivatives can serve as a valuable prototype for further development. The *in vivo* activity of title compounds against plant pathogenic bacteria is now in progress. Preliminary results indicate that further optimization of thiourea-based inhibitors might provide a new class of bactericides. Furthermore, additional experiments are also needed on human tyrosinase or melanocytes to support the potential of these compounds for human-directed applications.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.03.013.

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