Bioorganic & Medicinal Chemistry 23 (2015) 924–931

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Rational design, synthesis and structure–activity relationships of 4-alkoxy- and 4-acyloxy-phenylethylenethiosemicarbazone analogues as novel tyrosinase inhibitors

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ARTICLE INFO

Article history:
Received 26 November 2014
Revised 15 January 2015
Accepted 17 January 2015
Available online 22 January 2015

Keywords:
4-Alkoxy- and 4-acyloxy-phenylethylenethiosemicarbazone analogues
Tyrosinase inhibitor
SARs
Inhibition mechanism
Inhibitory kinetics

ABSTRACT

In continuing our program aimed to search for potent compounds as highly efficient tyrosinase inhibitors, here a series of novel 4-alkoxy- and 4-acyloxy-phenylethylenethiosemicarbazone analogues were designed, synthesized and their biological activities on mushroom tyrosinase were evaluated. Notably, most of compounds displayed remarkable tyrosinase inhibitory activities with IC_{50} value of lower than 1.0 μM. Furthermore, the structure–activity relationships (SARs) were discussed and the inhibition mechanism and the inhibitory kinetics of selected compounds **7k** and **8d** were also investigated. Taken together, these results suggested that such compounds could serve as the promising candidates for the treatment of tyrosinase-related disorders and further development of such compounds might be of great interest.

1. Introduction

Tyrosinase (EC 1.14.18.1; polyphenol oxidase, PPO), structurally belonging to the type-3 copper protein family, is widely distributed in animals, plants and microorganisms.[1] It is also well known that the enzyme is involved in the two-step oxidation for the transformation of L-tyrosine into dopaquinone, the key products for melanin pigment biosynthesis.[2] Due to its particularly prominent role in melanogenesis, tyrosinase has emerged in the past decades as a key target for the screening and the discovery of new inhibitors as the depigmenting agents.[3] Moreover, recently tyrosinase was also reported to link to the happen of Parkinson's and other neurodegenerative diseases,[4] the molting process of insects[5] and the browning of fruits and vegetables.[6] Therefore, tyrosinase inhibitors are clinically useful for the treatment of some dermatological disorders and also should have broad applications in food industry (antibrowning), agriculture (insecticide) and cosmetics (skin-lightening) due to decreasing the excessive accumulation of pigmentation resulting from the enzyme action.

As a result, a tremendously large number of natural and synthetic compounds acting as tyrosinase inhibitors have been reported to date.[3c,7,8] However, only few of them are put into practical use, largely because of the lack of their individual activities or safety concerns. Undoubtedly, more efforts are urgently needed to discover and develop new tyrosinase inhibitors with better activities and lower side effects.

With this in mind and inspired by the pioneering work of Luo[9] and Chen,[10] recently our groups[11] have intensively developed several series of aromatic aldehydes/ketones and their thiosemicarbazone derivatives as potent tyrosinase inhibitors. The SARs analysis revealed that (1) the introduction of a proper hydrophobic group at the position-4 of the phenyl ring was beneficial to tyrosinase inhibitory activity; (2) the thiosemicarbazone moiety was crucial for their potent tyrosinase inhibitory activities; and in general, the activity of methyl ketone thiosemicarbazone compounds was better than that of the corresponding aldehyde thiosemicarbazone compounds. Afterwards the elegant work from Boumendjel and Réglier groups demonstrated that the distance between the thiosemicarbazone moiety and the aromatic nucleus played an important role in determining the tyrosinase inhibitory potency.[12] More recently, it should be emphasized that Belle and co-workers defined in detail phenylmethylene thiosemicarbazone

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http://dx.doi.org/10.1016/j.bmc.2015.01.024
0968-0896/

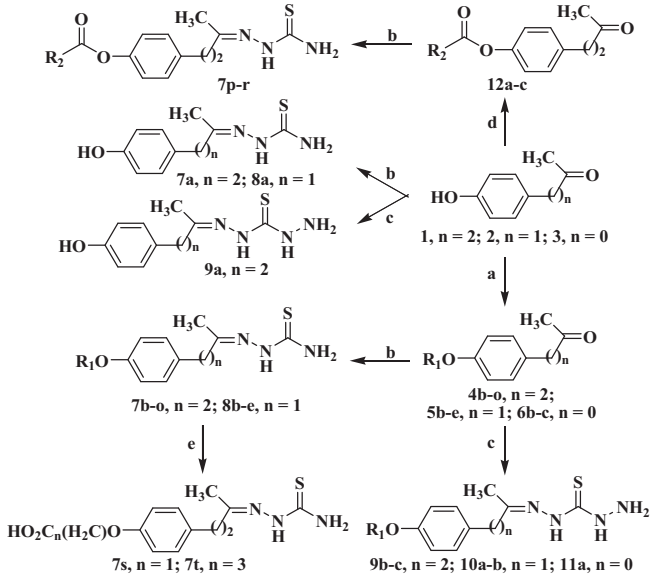
(PTSC) as tyrosinase inhibitor by combining enzymatic studies and coordination chemistry methods,[13] which opened a new avenue for the development of new and potent thiosemicarbazone-derived tyrosinase inhibitors.

Taking advantage of above information, we speculated that condensation products of thiosemicarbazide with 4-hydroxy- or 4-alkoxy-phenylketones bearing a proper length of methylene linker ($n = 0$, 1 or 2) between the phenyl ring and the ketone moiety might exhibit potent tyrosinase inhibitory activities. Therefore, in continuing our program aimed to search for potent compounds as tyrosinase inhibitors[11,14] and to better understand the structure–activity relationships (SARs) of thiosemicarbazone compounds, here a series of 4-alkoxy- and 4-acyloxy-phenylethylenethiosemicarbazone analogues were designed, synthesized and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were evaluated. To the best of our knowledge, this is the first time to report the tyrosinase inhibitory effects of such compounds. Moreover, to more clearly verify the importance of thiosemicarbazone group, the corresponding thiocarbonohydrazone analogues were synthesized and investigated. Besides, the inhibition mechanism and the inhibitory kinetics of selected compounds were also studied. We hope that these findings can lead to the discovery of potential pharmacological agents for treating the tyrosinase-related disorders and also offer key and useful information for future design of highly potent tyrosinase inhibitors. The synthetic procedure was outlined in Scheme 1, and the chemical structure of the corresponding substituent at the phenyl ring was given in Tables 1 and 2.

2. Experimental

2.1. General

Melting points were determined on a WRS-1B digital instrument without correction. NMR spectra were recorded on a Varian Mercury-Plus 300 spectrometer in DMSO-d_6. All chemical shifts (δ) were quoted in parts per million and coupling constants (J) were given in Hertz. Mass spectra were obtained from VG ZAB-HS, LCMS-2010A or LCQ DECA XP spectrometer. Elemental analyses were performed with a Vario EL cube instrument. All commercially available reagents and solvents were used without further purification. Mushroom tyrosinase (specific activity of the enzyme is 6680 U/mg) and L-DOPA (L-3,4-dihydroxyphenylalanine) were purchased from Sigma Chemical Co.



Scheme 1. Synthesis of thiosemicarbazone compounds (**7a–t** and **8a–e**) and thiocarbonohydrazone compounds (**9a–c**, **10a–b** and **11a**). Reagents and conditions: (a) R_1X (X = Br or I), K_2CO_3, anhydrous acetone, rt, 4–7 h; (b) thiosemicarbazide, anhydrous ethanol, acetic acid, 50–80 °C, 3 h; (c) thiocarbonohydrazide, anhydrous ethanol, acetic acid, 50–80 °C, 3 h; (d) triethylamine, R_2COCl, rt, 1–3 h; (e) 15% NaOH, ethanol, rt, 3–8 h.

2.2. Procedure for the synthesis of targeted thiosemicarbazone compounds 7a–t, 8a–e and thiocarbonohydrazone compounds 9a–c, 10a–b, 11a

2.2.1. Synthesis of 7a, 8a and 9a

A mixture of 4-(4-hydroxyphenyl)butan-2-one (**1**) or 1-(4-hydroxyphenyl)propan-2-one (**2**) or 1-(4-hydroxyphenyl)ethanone (**3**) (5.0 mmol), thiosemicarbazide or thiocarbonohydrazide (5.0 mmol) and acetic acid (0.5 mL) was stirred in anhydrous ethanol at 50–80 °C for 3 h. After completion of the reaction as indicated by TLC, the reaction mixture was cooled to room temperature and the precipitate solid was filtered and washed with ethanol to afford pure target compounds **7a**, **8a** and **9a**.

2.2.1.1. 4-(4-Hydroxyphenyl)butan-2-ylidenethiosemicarbazide (7a). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.90 (s, 1H), 9.09 (s, 1H), 8.01 (s, 1H), 7.41 (s, 1H), 6.98 (d, J = 7.5 Hz, 2H), 6.63 (d, J = 6.9 Hz, 2H), 2.69 (t, J = 7.4 Hz, 2H), 2.47 – 2.38 (m, 2H), 1.89 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.7, 156.0, 153.3, 129.9, 127.1, 115.2, 43.6, 38.7, 15.7. ESI-MS m/z = 236.1 $[M-1]^-$. It was identified with the reported data.[11a]

2.2.1.2. 1-(4-Hydroxyphenyl)propan-2-ylidenethiosemicarbazide (8a). Solid product, yield 69%, mp 155–156 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.98 (s, 1H), 9.27 (s, 1H), 8.09 (s, 1H), 7.60 (s, 1H), 7.03 (d, J = 8.2 Hz, 2H), 6.70 (d, J = 8.3 Hz, 2H), 3.39 (s, 2H), 1.79 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.7, 156.0, 153.3, 129.9, 127.1, 115.2, 43.6, 15.7. ESI-MS m/z = 222.1 $[M-1]^-$.

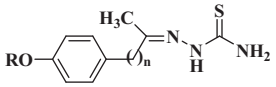
2.2.1.3. 4-(4-Hydroxyphenyl)butan-2-ylidenethiocarbonohydrazide (9a). Solid product, yield 69%, mp 157–158 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.19 (s, 2H), 9.12 (s, 1H), 6.98 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.3 Hz, 2H), 4.74 (s, 2H), 2.58–2.45 (m, 2H), 1.65–1.49 (m, 2H), 1.10 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 172.4, 155.2, 132.1, 129.1, 128.9, 115.0, 36.8, 28.5, 20.0. ESI-MS m/z = 253.1 $[M+1]^+$.

2.2.2. Synthesis of 7b–o, 8b–e, 9b–c, 10a–b and 11a

Into 50 mL of anhydrous acetone were added 10.0 mmol of compound **1** (**2** or **3**), 13.0 mmol of the corresponding alkyl bromide or alkyl iodide and 20.0 mmol of K_2CO_3, the above mixture was stirred at room temperature for 4–7 h. After completion of the reaction as indicated by TLC, the reaction mixture was filtered and the solvent was removed by evaporation at vacuum to get crude products, followed by chromatography to provide the pure intermediates **4b–o**, **5b–e** and **6b–c**. Then, they respectively reacted with thiosemicarbazide or thiocarbonohydrazide in the presence of acetic acid (0.5 mL) at 50–80 °C for 3 h to deliver the desired products **7b–o**, **8b–e**, **9b–c**, **10a–b** and **11a**.

2.2.2.1. 4-(4-Methoxyphenyl)butan-2-ylidenethiosemicarbazide (7b). Solid product, yield 88%, mp 143–144 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.93 (s, 1H), 8.03 (s, 1H), 7.43 (s, 1H), 7.14 (d, J = 8.5 Hz, 2H), 6.83 (d, J = 8.6 Hz, 2H), 3.71 (s, 3H), 2.81–2.72 (m, 2H), 2.55–2.45 (m, 2H), 1.91 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.5, 157.4, 153.6, 133.2, 129.2, 113.6, 54.9, 39.9, 30.6, 16.6. Anal. Calcd for $C_{12}H_{17}N_3OS$: C, 57.34; H, 6.82; N,

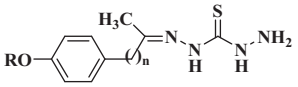
Table 1
Structures and the inhibitory activities against mushroom tyrosinase (diphenolase) of thiosemicarbazone compounds **7a–t** and **8a–e**



7a-t, n = 2; 8a-e,n = 1

Compd	R	*n*	IC_{50} (μM)	Compd	R	*n*	IC_{50} (μM)
7a	H	2	0.54[11a]	**7n**	$CH_2CO_2C_2H_5$	2	0.515
7b	CH_3	2	0.188	**7o**	$(CH_2)_3CO_2C_2H_5$	2	0.328
7c	C_2H_5	2	0.209	**7p**	$COCH_3$	2	0.072
7d	*n*-C_3H_7	2	0.264	**7q**	$COCH(CH_3)_2$	2	0.288
7e	*n*-C_4H_9	2	0.272	**7r**	COPh	2	0.156
7f	*n*-C_5H_{11}	2	0.412	**7s**	CH_2CO_2H	2	0.593
7g	$CH_2CH_2CH(CH_3)_2$	2	0.475	**7t**	$(CH_2)_3CO_2H$	2	0.678
7h	*n*-C_6H_{13}	2	0.754	**8a**	H	1	2.71
7i	*n*-C_8H_{17}	2	0.865	**8b**	CH_3	1	0.42[11a]
7j	$CH(C_2H_5)_2$	2	0.445	**8c**	*n*-C_5H_{11}	1	0.613
7k	CH_2Ph	2	0.217	**8d**	CH_2Ph	1	0.34
7l	CH_2PhBr-4	2	0.438	**8e**	$(CH_2)_3CO_2C_2H_5$	1	0.478
7m	$CH_2CH_2CH_2Ph$	2	0.835	Kojic acid			28.0

Table 2
Structures and the inhibitory activities against mushroom tyrosinase (diphenolase) of thiocarbonohydrazone compounds **9a–c**, **10a–b** and **11a**



9a-c, n = 2;10a-b, n = 1;11a, n = 0

Compd	R	*n*	IC_{50} (μM)	Compd	R	*n*	IC_{50} (μM)
9a	H	2	125.6	**10a**	CH_3	1	158.87
9b	CH_3	2	208.5	**10b**	*n*-C_4H_9	1	224.1
9c	*n*-C_4H_9	2	231.5	**11a**	CH_3	0	109.6
Thiocarbonohydrazide			178.3	Kojic acid			28.0

16.72. Found: C, 57.15; H, 6.84; N, 16.64. ESI-MS *m*/*z* = 252.2 $[M+1]^+$.

2.2.2.2. 4-(4-Ethoxyphenyl)butan-2-ylidenethiosemicarbazide (7c). Solid product, yield 86%, mp 140–141 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.94 (s, 1H), 8.03 (s, 1H), 7.44 (s, 1H), 7.11 (d, *J* = 5.7 Hz, 2H), 6.80 (d, *J* = 6.7, 2H), 3.92–3.99 (m, 2H), 2.82–2.69 (m, 2H), 2.48 (t, *J* = 8.9, 2H), 1.90 (s, 3H), 1.29 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.6, 153.7, 133.1, 129.1, 114.1, 62.8, 39.9, 30.6, 16.6, 14.7. Anal. Calcd for $C_{13}H_{19}N_3OS$: C, 58.84; H, 7.22; N, 15.83. Found: C, 58.83; H, 7.22; N, 15.83. ESI-MS *m*/*z* = 266.2 $[M+1]^+$.

2.2.2.3. 4-(4-Propoxyphenyl)butan-2-ylidenethiosemicarbazide (7d). Solid product, yield 89%, mp 116–117 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.94 (s, 1H), 8.03 (s, 1H), 7.44 (s, 1H), 7.11 (d, *J* = 5.7 Hz, 2H), 6.80 (d, *J* = 6.7, 2H), 3.87 (t, *J* = 6.5, 2H), 2.82–2.70 (m, 2H), 2.49 (t, *J* = 12.2, 2H), 1.89 (s, 3H), 1.78–1.61 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.8, 153.7, 133.1, 129.1, 114.2, 68.7, 39.9, 30.6, 22.0, 16.6, 10.4. Anal. Calcd for $C_{14}H_{21}N_3OS$: C, 60.18; H, 7.58; N, 15.04. Found: C, 60.11; H, 7.56; N, 14.99. ESI-MS *m*/*z* = 280.2 $[M+1]^+$.

2.2.2.4. 4-(4-Butoxyphenyl)butan-2-ylidenethiosemicarbazide (7e). Solid product, yield 88%, mp 108–109 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.94 (s, 1H), 8.04 (s, 1H), 7.44 (s, 1H), 7.11 (d, *J* = 8.5 Hz, 2H), 6.81 (d, *J* = 8.5 Hz, 2H), 3.90 (t, *J* = 8.5 Hz, 2H), 2.81–2.70 (m, 2H), 2.56–2.43 (m, 2H), 1.90 (s, 3H), 1.73–1.59 (m, 2H), 1.49–1.33 (m, 2H), 0.91 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.8, 153.7, 133.1, 129.1, 114.1, 67.0, 40.0, 30.8, 30.6, 18.7, 16.6, 13.7. Anal. Calcd for $C_{15}H_{23}N_3OS$: C, 61.40; H, 7.90; N, 14.32. Found: C, 61.27; H, 7.84; N, 14.40. ESI-MS *m*/*z* = 294.3 $[M+1]^+$.

2.2.2.5. 4-(4-Pentyloxyphenyl)butan-2-ylidenethiosemicarbazide (7f). Solid product, yield 88%, mp 117–118 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.94 (s, 1H), 8.03 (s, 1H), 7.44 (s, 1H), 7.11 (d, *J* = 8.5 Hz, 2H), 6.81 (d, *J* = 8.5 Hz, 2H), 3.90 (t, *J* = 8.5, 2H), 2.83–2.70 (m, 2H), 2.53–2.44 (m, 2H), 1.90 (s, 3H), 1.77–1.60 (m, 2H), 1.47–1.26 (m, 4H), 0.89 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.8, 153.7, 133.1, 129.1, 114.1, 67.2, 40.0, 30.6, 28.4, 27.7, 21.9, 16.6, 13.9. Anal. Calcd for $C_{16}H_{25}N_3OS$: C, 62.50; H, 8.20; N, 13.67. Found: C, 62.46; H, 8.13; N, 13.75. ESI-MS *m*/*z* = 308.3 $[M+1]^+$.

2.2.2.6. 4-(4-*iso*-Pentyloxyphenyl)butan-2-ylidenethiosemicarbazide (7g). Solid product, yield 89%, mp 110–111 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.94 (s, 1H), 8.03 (s, 1H), 7.44 (s, 1H), 7.11 (d, *J* = 8.4 Hz, 2H), 6.82 (d, *J* = 8.4 Hz, 2H), 3.93 (t, *J* = 8.7, 2H), 2.82–2.70 (m, 2H), 2.56–2.46 (m, 2H), 1.89 (s, 3H), 1.80–1.69 (m, 1H), 1.61–1.54 (m, 2H), 0.91 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.8, 153.7, 133.1, 129.1, 114.2, 65.7, 40.0, 37.5, 30.6, 24.5, 22.4, 16.6. Anal. Calcd for $C_{16}H_{25}N_3OS$: C, 62.50; H, 8.20; N, 13.67. Found: C, 62.56; H, 8.15; N, 13.67. ESI-MS *m*/*z* = 308.3 $[M+1]^+$.

2.2.2.7. 4-(4-Hexyloxyphenyl)butan-2-ylidenethiosemicarbazide (7h). Solid product, yield 88%, mp 111–112 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.95 (s, 1H), 8.05 (s, 1H), 7.44 (s, 1H), 7.11 (d, *J* = 8.6 Hz, 2H), 6.81 (d, *J* = 8.5 Hz, 2H), 3.90 (t, *J* = 8.5, 2H), 2.82–2.71 (m, 2H), 2.55–2.46 (m, 2H), 1.91 (s, 3H), 1.75–1.60 (m, 2H), 1.46–1.23 (m, 6H), 0.87 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.8, 153.6, 133.1, 129.1, 114.1, 67.3, 40.0, 31.0, 30.7, 28.7, 25.2, 22.1, 16.7, 13.9. Anal. Calcd for $C_{17}H_{27}N_3OS$: C, 63.51; H, 8.47; N, 13.07. Found: C, 53.28; H, 8.39; N, 13.07. ESI-MS *m*/*z* = 322.2 $[M+1]^+$.

2.2.2.8. 4-(4-Octyloxyphenyl)butan-2-ylidenethiosemicarbazide (7i). Solid product, yield 82%, mp 90–92 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.95 (s, 1H), 8.05 (s, 1H), 7.44 (s, 1H), 7.11 (d, *J* = 8.6 Hz, 2H), 6.80 (d, *J* = 8.5 Hz, 2H), 3.90 (t, *J* = 8.5, 2H), 2.81–2.69 (m, 2H), 2.50 (t, *J* = 5.5 Hz, 2H), 1.91 (s, 3H), 1.75–1.61

(m, 2H), 1.46–1.17 (m, 10H), 0.86 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.8, 153.6, 133.1, 129.1, 114.1, 67.3, 40.0, 31.2, 30.7, 28.7, 28.7, 28.7, 25.5, 22.1, 16.6, 13.9. Anal. Calcd for $C_{19}H_{31}N_3OS$: C, 65.29; H, 8.94; N, 12.02. Found: C, 65.22; H, 8.82; N, 12.01. ESI-MS m/z = 350.2 $[M+1]^+$.

2.2.2.9. 4-(4-(Pentan-3-yl)oxyphenyl)butan-2-ylidenethiosemicarbazide (7j). Solid product, yield 88%, mp 111–112 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.95 (s, 1H), 8.06 (s, 1H), 7.46 (s, 1H), 7.10 (d, J = 8.6 Hz, 2H), 6.81 (d, J = 8.5 Hz, 2H), 4.17–4.10(m, 1H), 2.81–2.69 (m, 2H), 2.57–2.46 (m, 2H), 1.91 (s, 3H), 1.66–1.50 (m, 4H), 0.88 (t, J = 7.4 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.5, 156.4, 153.6, 133.1, 129.2, 115.5, 79.0, 40.0, 38.5, 30.6, 25.5, 16.7, 9.3. Anal. Calcd for $C_{16}H_{25}N_3OS$: C, 62.50; H, 8.20; N, 13.67. Found: C, 62.55; H, 8.11; N, 13.69. ESI-MS m/z = 308.3 $[M+1]^+$.

2.2.2.10. 4-(4-Benzyloxyphenyl)butan-2-ylidenethiosemicarbazide (7k). Solid product, yield 92%, mp 136–137 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.96 (s, 1H), 8.06 (s, 1H), 7.50–7.27 (m, 6H), 7.14 (d, J = 8.6 Hz, 2H), 6.90 (d, J = 6.7, 2H),, 5.05 (s, 2H), 2.83–2.72 (m, 2H), 2.54–2.46 (m, 2H), 1.90 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.5, 153.6, 137.2, 133.5, 129.2, 128.4, 127.7, 127.6, 114.5, 69.1, 39.9, 30.6, 16.7. Anal. Calcd for $C_{18}H_{21}NOS$: C, 66.02; H, 6.46; N, 12.83. Found: C, 66.12; H, 6.49; N, 12.81. ESI-MS m/z = 328.3 $[M+1]^+$.

2.2.2.11. 4-(4-(4-BromoBenzyloxy)phenyl)butan-2-ylidenethiosemicarbazide (7l). Solid product, yield 90%, mp 174–175 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.91 (s, 1H), 8.02 (s, 1H), 7.56 (d, J = 8.3 Hz, 2H), 7.37 (d, J = 7.8 Hz, 3H), 7.11 (d, J = 8.2 Hz, 2H), 6.87 (d, J = 7.9 Hz, 2H), 5.03 (s, 2H), 2.81–2.70 (m, 2H), 2.49–2.44 (m, 2H), 1.89 (s, 3H).^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.3, 153.6, 136.7, 133.7, 131.3, 129.7, 129.2, 120.8, 114.6, 68.3, 39.9, 30.6, 16.7. Anal. Calcd for $C_{18}H_{20}BrN_3OS$: C, 53.20; H, 4.96; N, 10.34. Found: C, 53.22; H, 4.95; N, 10.25. SI-MS m/z = 406.1 $[M+1]^+$.

2.2.2.12. 4-(4-(3-Phenylpropyl)phenyl)butan-2-ylidenethiosemicarbazide (7m). Solid product, yield 78%, mp 131–132 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.95 (s, 1H), 8.05 (s, 1H), 7.44 (s, 1H), 7.33–7.17 (m, 5H), 7.12 (d, J = 8.6 Hz, 2H), 6.82 (d, J = 6.7, 2H), 3.90 (t, J = 8.5, 2H), 2.82–2.68 (m, 4H), 2.55–2.44 (m, 2H), 2.05–1.94 (m, 2H), 1.91 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 156.7, 153.6, 141.4, 133.2, 129.2, 128.3 (overlapped peak), 125.8, 114.2, 66.5, 40.0, 31.5, 30.7, 30.4, 16.7. Anal. Calcd for $C_{20}H_{25}N_3OS$: C, 67.57; H, 7.09; N, 11.82. Found: C, 67.49; H, 7.07; N, 11.81. ESI-MS m/z = 256.2 $[M+1]^+$.

2.2.2.13. 4-(4-Ethoxycarbonylmethylphenyl)butan-2-ylidenethiosemicarbazide (7n). Solid product, yield 85%, mp 124–125 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.95 (s, 1H), 8.05 (s, 1H), 7.44 (s, 1H), 7.14 (d, J = 8.7 Hz, 2H), 6.82 (d, J = 8.4 Hz, 2H), 4.72 (s, 2H), 4.20–4.12 (m, 2H), 2.84–2.72 (m, 2H), 2.55–2.44 (m, 2H), 1.91 (s, 3H), 1.20 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 168.8, 155.8, 153.6, 134.1, 129.2, 114.3, 64.7, 60.5, 39.9, 30.6, 16.7, 14.0. Anal. Calcd for $C_{15}H_{21}N_3O_3S$: C, 55.71; H, 6.54; N, 12.99. Found: C, 55.95; H, 6.51; N, 13.06. ESI-MS m/z = 324.3 $[M+1]^+$.

2.2.2.14. 4-(4-(3-Ethoxycarbonylpropyl)phenyl)butan-2-ylidenethiosemicarbazide (7o). Solid product, yield 85%, mp 94–95 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.95 (s, 1H), 8.05 (s, 1H), 7.44 (s, 1H), 7.12 (d, J = 8.6 Hz, 2H), 6.81 (d, J = 8.6 Hz, 2H), 4.10–4.03 (m, 2H), 3.99–3.88 (m, 2H), 2.82–2.70 (m, 2H), 2.55–2.38 (m, 4H), 2.01–1.86 (m, 5H), 1.17 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 172.5, 156.6, 153.6, 133.3, 129.2, 114.2, 66.3, 59.8, 40.0, 30.6, 30.1, 24.2, 16.6, 14.1. Anal. Calcd for $C_{17}H_{25}N_3O_3S$: C, 58.09; H, 7.17; N, 11.96. Found: C, 58.06; H, 7.18; N, 12.04. ESI-MS m/z = 352.3 $[M+1]^+$.

2.2.2.15. 1-(4-Methoxyphenyl)propan-2-ylidenethiosemicarbazide (8b). Solid product. ^{1}H NMR (300 MHz, DMSO) δ 9.97 (s, 1H), 8.07 (s, 1H), 7.59 (s, 1H), 7.13 (d, J = 7.4 Hz, 2H), 6.85 (d, J = 7.0 Hz, 2H), 3.71 (s, 3H), 3.43 (s, 2H), 1.79 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.6, 158.0, 153.1, 130.0, 129.0, 114.0, 55.0, 43.6, 15.8. It was identified with the reported data.[11a]

2.2.2.16. 1-(4-Pentyloxyphenyl)propan-2-ylidenethiosemicarbazide (8c). Solid product, yield 81%, mp 137–139 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.00 (s, 1H), 8.10 (s, 1H), 7.62 (s, 1H), 7.14 (d, J = 7.8 Hz, 2H), 6.86 (d, J = 7.7 Hz, 2H), 3.91 (t, J = 6.4 Hz, 2H), 3.44 (s, 2H), 1.80 (s, 3H), 1.76–1.65 (m, 2H), 1.42–1.27 (m, 4H), 0.89 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.7, 157.4, 153.1, 129.9, 128.8, 114.4, 67.3, 43.6, 28.4, 27.7, 21.9, 15.7, 13.9. Anal. Calcd for $C_{15}H_{23}N_3OS$: C, 61.40; H, 7.90; N, 14.32. Found: C, 61.15; H, 7.81; N, 14.41. ESI-MS m/z = 294.3 $[M+1]^+$.

2.2.2.17. 1-(4-Benzyloxyphenyl)propan-2-ylidenethiosemicarbazide (8d). Solid product, yield 83%, mp 153–154 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.00 (s, 1H), 8.10 (s, 1H), 7.62 (s, 1H), 7.48–7.31 (m, 5H), 7.17 (d, J = 8.6 Hz, 2H), 6.96 (d, J = 9.0, 2H), 5.07 (s, 2H), 3.43 (s, 2H), 1.80 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.7, 157.1, 153.1, 137.1, 130.0, 129.2, 128.4, 127.8, 127.7, 114.8, 69.1, 43.6, 15.8. Anal. Calcd for $C_{17}H_{19}N_3OS$: C, 65.15; H, 6.11; N, 13.41. Found: C, 65.32; H, 6.10; N, 13.01. ESI-MS m/z = 314.1 $[M+1]^+$.

2.2.2.18. 1-(4-(3-Ethoxycarbonyl)propoxyphenyl)proptan-2-ylidenethiosemicarbazide (8e). Yellowish solid product, yield 78%, mp 93–94 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.99 (s, 1H), 8.09 (s, 1H), 7.62 (s, 1H), 7.15 (d, J = 8.6 Hz, 2H), 6.86 (d, J = 8.6 Hz, 2H), 4.10–4.01 (m, 2H), 3.95 (t, J = 6.3 Hz, 2H), 3.44 (s, 2H), 2.44 (t, J = 7.3 Hz, 2H), 2.00–1.91 (m, 2H), 1.80 (s, 3H), 1.17 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.7, 172.5, 157.2, 153.1, 129.9, 129.0, 114.4, 66.4, 59.8, 43.6, 30.1, 24.2, 15.7, 14.1. ESI-MS m/z = 338.3 $[M+1]^+$.

2.2.2.19. 4-(4-Methoxyphenyl)butan-2-ylidenethiocarbonohydrazide (9b). Solid product, yield 79%, mp 167–168 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.16 (s, 2H), 7.07 (d, J = 7.9 Hz, 2H), 6.81 (d, J = 7.5 Hz, 2H), 4.73 (s, 2H), 3.69 (s, 3H), 2.52 (t, J = 9.6 Hz, 2H), 1.56 (t, J = 8.9 Hz, 2H), 1.09 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.8, 160.1, 148.4, 130.4, 128.7, 114.4, 67.7, 31.2, 19.2, 14.2. ESI-MS m/z = 267.0 $[M+1]^+$.

2.2.2.20. 4-(4-Butoxyphenyl)butan-2-ylidenethiocarbonohydrazide (9c). Solid product, yield 73%, mp 147–148 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.15 (s, 2H), 7.05 (d, J = 7.2 Hz, 2H), 6.80 (d, J = 7.2 Hz, 2H), 4.72 (s, 2H), 3.89 (t, J = 6.1 Hz, 2H), 2.50 (s, 2H), 1.74 – 1.60 (m, 2H), 1.58–1.49 (m, 2H), 1.41 (m, 2H), 1.09 (s, 3H), 0.91 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 173.0, 157.3, 134.3, 129.7, 129.5, 114.7, 67.5, 65.6, 31.3, 28.8, 20.5, 19.2, 14.2. ESI-MS m/z = 309.1 $[M+1]^+$.

2.2.2.21. 1-(4-Methoxyphenyl)propan-2-ylidenethiocarbonohydrazide (10a). White crystals, yield 74%, mp 179–180 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.97 (s, 1H), 9.38 (s, 1H), 7.15 (d, J = 7.7 Hz, 2H), 6.85 (d, J = 7.4 Hz, 2H), 4.81 (s, 2H), 3.71 (s, 3H), 3.43 (s, 2H), 1.76 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6)

δ: 172.9, 158.4, 132.1, 132.0, 129.4, 113.5, 66.2, 55.4, 20.2. ESI-MS m/z = 252.9 $[M+1]^+$.

2.2.2.22. 1-(4-Butoxyphenyl)propan-2-ylidenethiocarbonohydrazide (10b). Solid product, yield 77%, mp 168–169 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.24 (s, 2H), 7.14 (d, J = 8.0 Hz, 2H), 6.79 (d, J = 7.9 Hz, 2H), 4.79 (s, 2H), 3.92 (t, J = 6.3 Hz, 2H), 2.56 (s, 2H), 1.75–1.60 (m, 2H), 1.50–1.37 (m, 2H), 0.94 (t, J = 7.2 Hz, 3H), 0.87 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 172.8, 157.8, 132.1, 132.0, 128.9, 114.0, 67.4, 66.2, 31.3, 20.2, 19.2, 14.2. ESI-MS m/z = 295.1 $[M+1]^+$.

2.2.2.23. 1-(4-Methoxyphenyl)ethylidenethiocarbonohydrazide (11a). Solid product, yield 75%, mp 179–180 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.09 (s, 1H), 9.63 (s, 1H), 7.91 (d, J = 8.3 Hz, 2H), 6.91 (d, J = 8.2 Hz, 2H), 4.91 (s, 2H), 3.79 (s, 3H), 2.25 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.8, 160.6, 148.4, 128.7, 128.4, 114.0, 68.3, 55.7. ESI-MS m/z = 238.9 $[M+1]^+$.

2.2.3. Synthesis of 7p–r

To a mixture of 10.0 mmol of 4-(4-hydroxyphenyl)butan-2-one (**1**) and 30.0 mmol of triethylamine in 50 mL of dry dichloromethane, 10.0 mmol of the corresponding acyl chloride was added dropwise over 10 min. After stirring at room temperature for 1–3 h, the mixture was poured into 100 mL of ice-water. The organic phase was washed with 5% $NaHCO_3$ and water, dried over anhydrous Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography to give the intermediates **12a–c**, which then reacted with thiosemicarbazide in the presence of acetic acid (0.5 mL) at 50–80 °C for 3 h to deliver the desired products **7p–r**.

2.2.3.1. 4-(4-Acetoxyphenyl)butan-2-ylidenethiosemicarbazide (7p). Solid product, yield 93%, mp 141–142 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.96 (s, 1H), 8.05 (s, 1H), 7.47 (s, 1H), 7.26 (d, J = 6.8 Hz, 2H), 7.01 (d, J = 6.6 Hz, 2H), 2.84 (t, J = 7.6 Hz, 2H), 2.59–2.47 (m, 2H), 2.24 (s, 3H), 1.93 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.5, 169.2, 153.4, 148.5, 138.9, 129.1, 121.5, 39.9, 30.8, 20.8, 16.7. ESI-MS m/z = 302.1[M+Na]$^+$.

2.2.3.2. 4-(4-*iso*-Butylacyloxyphenyl)butan-2-ylidenethiosemicarbazide (7q). Solid product, yield 89%, mp 140–141 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.96 (s, 1H), 8.04 (s, 1H), 7.47 (s, 1H), 7.26 (d, J = 8.5 Hz, 2H), 7.00 (d, J = 8.7, 2H), 2.90–2.75 (m, 3H), 2.59–2.51 (m, 2H), 1.92 (s, 3H), 1.22 (d, J = 6.8 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.5, 175.1, 153.4, 148.6, 138.9, 129.2, 121.4, 39.2, 33.3, 30.8, 18.7, 16.7. ESI-MS m/z = 308.1 $[M+1]^+$.

2.2.3.3. 4-(4-Benzoxyphenyl)butan-2-ylidenethiosemicarbazide (7r). Solid product, yield 91%, mp 171–172 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.98 (s, 1H), 8.13 (d, J = 8.4 Hz, 2H), 8.07 (s, 1H), 7.75 (t, J = 7.4 Hz, 1H), 7.61 (t, J = 7.5 Hz, 2H), 7.50 (s, 1H), 7.33 (d, J = 8.4 Hz, 2H), 7.19 (d, J = 8.5 Hz, 2H), 2.94–2.85 (m, 2H), 2.59–2.54 (m, 2H), 1.95 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.5, 164.6, 153.4, 148.7, 139.2, 134.0, 129.7, 129.3, 129.0, 128.9, 121.6, 39.6, 30.8, 16.7. ESI-MS m/z = 342.1 $[M+1]^+$.

2.2.4. Synthesis of 7s and 7t

A mixture of 10.0 mmol of **7n** or **7o** and 3.0 mL of 15% NaOH in 50 mL of ethanol was stirred at room temperature for 3–8 h. After completion of the reaction as indicated by TLC, the reaction mixture was neutralized with 5% HCl, and the solvent was removed under reduced pressure. The obtained residue was purified by column chromatography to provide the desired products **7s–t**.

2.2.4.1. 4-(4-Carboxylmethylphenyl)butan-2-ylidenethiosemicarbazide (7s). Solid product, yield 89%, mp 183–184 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.96 (s, 1H), 9.95 (s, 1H), 8.03 (s, 1H), 7.44 (s, 1H), 7.13 (d, J = 8.7 Hz, 2H), 6.80 (d, J = 8.7, 2H), 4.62 (s, 2H), 2.79–2.74 (m, 2H), 2.53–2.47 (m, 2H), 1.91 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.6, 170.2, 156.0, 153.5, 133.6, 128.9, 113.6, 64.4, 39.9, 30.4, 16.2. ESI-MS m/z = 296.1 $[M+1]^+$.

2.2.4.2. 4-(4-(3-Carboxylpropyl)phenyl)butan-2-ylidenethiosemicarbazide (7t). Solid product, yield 86%, mp 152–153 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.14 (s, 1H), 9.95 (s, 1H), 8.05 (s, 1H), 7.45 (s, 1H), 7.12 (d, J = 8.7 Hz, 2H), 6.82 (d, J = 8.5 Hz, 2H), 3.93 (t, J = 6.3 Hz, 2H), 2.82–2.72 (m, 2H), 2.53–2.43 (m, 2H), 2.37 (t, J = 7.3 Hz, 2H), 1.98–1.86 (m, 5H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 174.1, 156.6, 153.6, 133.3, 129.2, 114.2, 66.4, 39.9, 30.7, 30.1, 24.3, 16.6. ESI-MS m/z = 346.1[M+Na]$^+$.

2.3. Assay of inhibition activity of target compounds

The inhibition of target compounds on the diphenolase activity of mushroom tyrosinase was investigated by our reported procedure[11,14] with slight modifications. Briefly, all the synthesized compounds were screened for the diphenolase inhibitory activity against tyrosinase using L-DOPA as substrate. All the thiosemicarbazone and thiocarbonohydrazone compounds were dissolved in DMSO and their final concentration in DMSO was 2.0%. Phosphate buffer, pH 6.8, was used to dilute the DMSO stock solution of test compounds. Thirty units of tyrosinase (0.5 mg/mL) was first pre-incubated with the samples, in 50 mM phosphate buffer (pH = 6.8), for 10 min at 25 °C, L-DOPA solution (0.5 mM) was added to the mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm of formation of DOPAchrome for 1 min. The measurement was completed in triplicate for each concentration and averaged before further calculation. IC_{50} value was determined by interpolation of the dose-response curves. Here kojic acid was used as a control and its IC_{50} value was also determined.

3. Results and discussion

3.1. Synthesis

As shown in Scheme 1, 4-(4-hydroxyphenyl)butan-2-one (**1**), 1-(4-hydroxyphenyl)propan-2-one (**2**) and 1-(4-hydroxyphenyl) ethanone (**3**) could be alkylated smoothly by using a simple procedure to give 4-(4-alkoxyphenyl)butan-2-one (**4b–o**), 1-(4-alkoxyphenyl)propan-2-one (**5b–e**) and 1-(4-alkoxyphenyl)ethanone (**6b–c**). These ketone compounds reacted with thiosemicarbazide or thiocarbonohydrazide in absolute ethanol to provide the corresponding compounds **7a–o**, **8a–e**, **9a–c**, **10a–b** and **11a** in good to excellent yields. All the target compounds were characterized by chemical and spectral methods.

The reaction of 4-(4-hydroxyphenyl)butan-2-one (**1**) and acyl chloride could be carried out to afford compounds **12a–c**, which reacted with thiosemicarbazide to afford thiosemicarbazone compounds **7p–r**.

Compounds **7s** and **7t** were prepared respectively by the hydrolysis of compounds **7n** and **7o** in the presence of 15% NaOH in ethanol at room temperature.

3.2. Biological activity

3.2.1. Inhibitory effects on the o-diphenolase activity of tyrosinase

All the synthesized compounds were subjected to mushroom tyrosinase inhibition assay by using L-DOPA as substrate, according

to the method described by our previous reports with slight modifications.[11,14] The resulted showed that all the target 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}). Here the well-known tyrosinase inhibitor kojic acid was used as the standard reference. The IC_{50} values of all the compounds were summarized in Tables 1 and 2. To our delight, the results from Table 1 showed that most of compounds delivered remarkable tyrosinase inhibitory activities with IC_{50} value of lower than 1.0 μM, revealing that such thiosemicarbazone compounds were worthy to be investigated further for the application in medicine, agriculture, cosmetics and food industry.

3.2.2. Structure–activity relationships (SARs) analysis

From the data of the tyrosinase inhibitory activities of our synthetic 4-alkoxy- and 4-actyloxy-phenylethylenethiosemicarbazone analogues, the following SAR results could be derived:

(1) All the compounds demonstrated highly efficient tyrosinase inhibitory activities, while their corresponding parent methyl ketones had no obvious inhibitory effects on tyrosinase (the data not shown). The results suggested that the thiosemicarbazone moiety played a very key role in determining the tyrosinase inhibitory activity, which further supported previous reports by Luo,[9] Chen[10] and our groups.[11]

(2) As shown in Table 1, the results showed that the compounds bearing the corresponding alkoxy or acyloxy groups at the position-4 of the phenyl ring generally showed better tyrosinase inhibitory activities than 4-OH substituted phenylethylenethiosemicarbazone. These results indicated that the introduction of proper structure-based hydrophobic subunit into the position-4 of the phenyl ring could efficiently enhance tyrosinase inhibitory activity, which confirmed our speculation. Moreover, the hydrolyzed products **7s** and **7t** from compounds **7n** and **7o**, respectively, had no contribute to tyrosinase inhibitory activities, suggesting that the hydrogen-bond interaction might not be formed between the carboxyl group of **7s** and **7t** and the hydrophobic protein pocket of tyrosinase.

(3) In general, the tyrosinase inhibitory activity was deceased gradually with the elongation of alkyl chain. The results revealed that the introduction of the long and/or large-size alkyl groups might retard the binding of inhibitor and the active site of tyrosinase, thus leading to a decrease of tyrosinase inhibitory activity.

(4) It was observed that compounds **7f** and **8c** demonstrated high tyrosinase activities with similar IC_{50} values (for **7f**, 0.412 μM; for **8c**, 0.613 μM). In comparison with **7k** and **8d**, the same finding was also obtained. These results hinted that, in the present investigation, the length of methylene linker between the phenyl ring and the thiosemicarbazone moiety had not obvious influence on the tyrosinase inhibitory activity.

(5) When an amino group was introduced to thiosemicarbazide part, the obtained thiocarbonohydrazone compounds **9a–c**, **10a–b** and **11a** demonstrated a decrease sharply in the tyrosinase inhibitory activities (Table 2), showing that the introduction of thiocarbonohydrazone moiety was unfavorable for the tyrosinase inhibitory activity, although the inhibitory activity of thiocarbonohydrazide (IC_{50} = 178.3 μM) was higher than that of thiosemicarbazide (IC_{50} = 2000 μM). Meanwhile, the present results further confirmed the particularly prominent role of thiosemicarbazone moiety in determining the tyrosinase inhibitory potency.

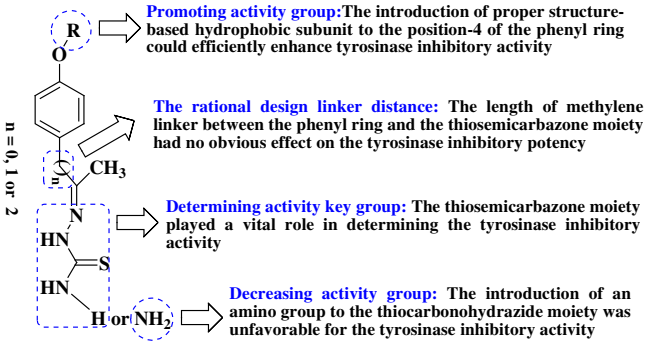


Figure 1. SARs analysis for the inhibitory activities of the target compounds on mushroom tyrosinase

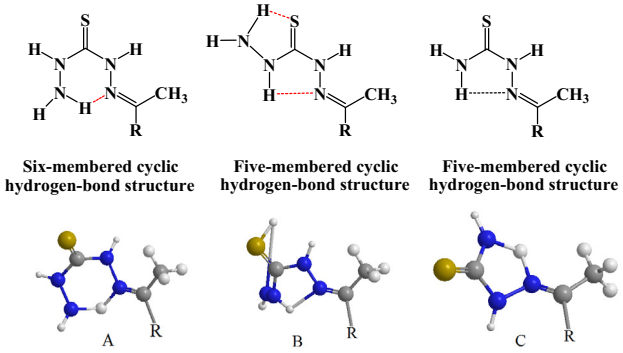


Figure 2. The proposed forms (plane hydrogen-bond structures and three-dimensional structures) of thiocarbonohydrazone compounds (**A** and **B**) and thiosemicarbazone compounds (**C**). [reference: ChemBioDraw Ultra 11.0. PerkinElmer: Waltham, MA, USA, 2013. Available online: http://www.cambridgesoft.com (accessed on 21 November 2013).].

The above SAR results were summarized and illustrated in Figure 1.

3.2.3. Defining the difference of the tyrosinase inhibitory activities of thiosemicarbazones and thiocarbonohydrazones

According to the theory of the molecular structure, organic molecules would form intramolecular hydrogen bonds if possible. For the synthesized thiocarbonohydrazones, they could form two kinds of intramolecular hydrogen bonds that were demonstrated in forms **A** and **B**, respectively (Fig. 2). As shown in Fig. 2, it was observed that there are two intramolecular hydrogen bonds in form **B**, thus making form **B** possess a lower molecular energy than form **A**. Based on it, the molecules of thiocarbonohydrazone compounds should employ form **B** as a dominant structure. For the well-known thiosemicarbazones, they adopted a five-membered cyclic hydrogen-bond structure demonstrated in form **C**.[11a] By analysis of their three-dimensional models, we found that thiocarbonohydrazone compounds possess a larger space volume than thiosemicarbazone compounds. Such large size would obviously hinder thiocarbonohydrazone compounds to form the potential interaction with the active site of tyrosinase.

Moreover, it was well known that thiosemicarbazone compounds could have highly efficient tyrosinase inhibitory activities because their sulfur atoms on thiosemicarbazone moiety were able to efficiently complex the two copper ions in the active center of tyrosinase.[9–13] However, as shown in dominant form **B**, two five-number cycles were stably formed by the intramolecular hydrogen-bond interaction, and thus even if such compounds could touch the active center of tyrosinase, the existence of S–H intramolecular hydrogen-bond in thiocarbonohydrazone compounds

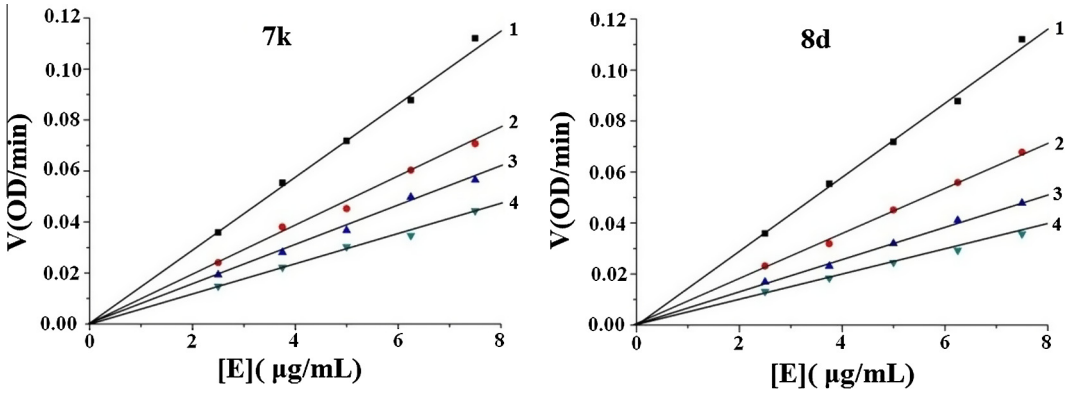


Figure 3. Determination of the inhibitory effects of **7k** and **8d** on mushroom tyrosinase for the oxidation of L-DOPA. The concentrations of compound **7k** for curves 1–4 were 0, 0.1, 0.2 and 0.3 μM, respectively. The concentrations of compound **8d** for curves 1–4 were 0, 0.2, 0.4 and 0.6 μM, respectively.

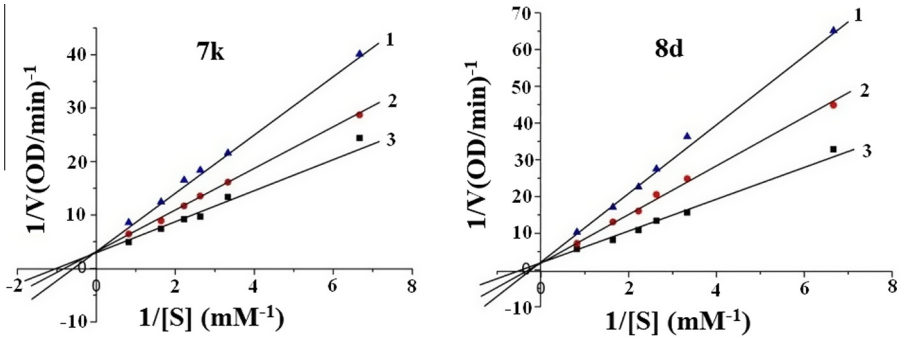


Figure 4. Determination of the inhibitory types of selected compounds **7k** and **8d** on mushroom tyrosinase for the oxidation of L-DOPA. The concentrations of **7k** for curves 1–3 were 0.3, 0.2 and 0.1 μM, respectively. The concentrations of **8d** for curves 1–3 were 0.6, 0.4 and 0.2 μM, respectively.

would dramatically decrease the chance of the interaction between the sulfur atom of thiosemicarbazone moiety and the dicopper ions in the active center of tyrosinase.

Therefore, thiocarbonohydrazone compounds presented weaker tyrosinase inhibitory activities than thiosemicarbazone compounds.

3.2.4. Inhibition mechanism of selected compounds 7k and 8d on mushroom tyrosinase

The inhibition mechanism of compounds **7k** and **8d** on mushroom tyrosinase for the oxidation of L-DOPA was first determined. Figure 3 showed the relationship between enzyme activity and concentration in the presence of different concentrations of compounds **7k** and **8d**, respectively. The results demonstrated that the plots gave a family of straight lines, which all passed through the origin. Increasing the inhibitor concentration resulted in a decrease in the slope of the line, indicating that the inhibition of compounds **7k** and **8d** on mushroom tyrosinase were reversible.

3.2.5. Inhibitory types of selected compounds 7k and 8d on mushroom tyrosinase

To further insight into the inhibition mechanism, finally the inhibitory types of the selected compounds **7k** and **8d** on mushroom tyrosinase for the oxidation of L-DOPA was determined by the Line Weaver–Burk double reciprocal plots. Figure 4 showed the double-reciprocal plots of the enzyme inhibited by compounds **7k** and **8d**. The results displayed that the plots of $1/V$ versus $1/[S]$ gave three straight lines with different slopes, but they intersected one another at the ordinate. The values of V_{max} remained the same and the values of K_m increased with increasing concentrations of the inhibitor, which indicated that compounds **7k** and **8d** were the competitive inhibitors of tyrosinase.[8c,15] The results revealed that **7k** and **8d** could only bind with the free enzyme.

Recently the crystallographic structure of tyrosinase from different species has been determined, enabling a close look at its three-dimensional structure and a better understanding of its mechanism of action.[16] On the basis of these and our obtained results, a possible binding mode was postulated. First, the sulfur atom of the thiosemicarbazide moiety efficiently formed strong chelation with the binuclear copper of tyrosinase. This interaction acted as a bridge to link the alkoxy (or acyloxy) group and the hydrophobic protein pocket, which facilitated the alkoxy (or acyloxy) moiety to interact with the hydrophobic active site of tyrosinase. The two potential interactions with both dicopper ions and the hydrophobic active site made the compounds perfectly occupy the active site of tyrosinase, which resulted in inhibiting the combination of the substrate L-DOPA and the binuclear copper catalytic site. As a consequence, it could be observed that these compounds exhibited such inhibitory effects on tyrosinase by acting as competitive inhibitors.

4. Conclusion

In summary, we have developed a series of 4-alkoxy- and 4-acyloxy-phenylethylenethiosemicarbazone analogues as novel tyrosinase inhibitors. The results showed that most of compounds exhibited remarkable tyrosinase inhibitory activities with IC_{50} value of lower than 1.0 μM. SARs analysis suggested that: (1) the

thiosemicarbazone moiety played a very key role in determining the tyrosinase inhibitory activity; (2) the introduction of proper structure-based hydrophobic subunit to the position-4 of the phenyl ring obviously enhanced tyrosinase inhibitory activity; (3) the length of alkyl chain attached on the position-4 of the phenyl ring influenced the tyrosinase inhibitory activity; (4) however, the length of methylene linker between the phenyl ring and the thiosemicarbazo moiety had no obvious effect on the tyrosinase inhibitory potency; (5) the introduction of thiocarbonohydrazide moiety was unfavorable for the tyrosinase inhibitory activity since the three-dimensional model analysis showed that the sulfur atom of thiocarbonohydrazide moiety has been involved in the formation of intramolecular hydrogen bond. Moreover, the inhibition mechanism and inhibition kinetics study revealed that these compounds exhibited such inhibitory effects on tyrosinase by acting as the reversible and competitive inhibitors. Taken together, these results presented here suggested that these molecules could serve as the interesting candidates for the treatment of tyrosinase-related disorders and as the lead compounds for the development of new and potent tyrosinase inhibitors. Therefore, further studies of these compounds using human tyrosinase and a human melanoma cell line are underway in our laboratory, and the research results will be reported in due course.

Acknowledgments

This work was supported by the Science and Technology Project of Guangdong Province, China (2009B060700048), Shanghai Municipal Natural Science Foundation, China (15ZR1447800) and the Fundamental Research Funds for the Central Universities.

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