Rational Design and Synthesis of 4-*O*-Substituted Phenylmethylenethiosemicarbazones as Novel Tyrosinase Inhibitors

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In continuing our program aimed to search for tyrosinase inhibitors, a series of novel 4-*O*-substituted phenylmethylenethiosemicarbazones were rational designed, synthesized and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were also evaluated. A fair number of compounds were found to have significant tyrosinase inhibitiory activity. Particularly, the IC_{50} values of compounds 3a—g, 3j and 3s were of the same magnitude as tropolone, one of the best tyrosinase inhibitors known so far. Furthermore, the structure–activity relationships of these compounds were also investigated. All these data suggested that these molecules might be utilized for the development of new candidate for the treatment of dermatological disorders, and further development of such compounds may be of interest.

Key words synthesis; tyrosinase inhibitory activity; the structure–activity relationship; 4-*O*-substituted phenylmethylenethiosemicarbazone

Tyrosinase or polyphenol oxidase (EC 1.14.18.1), a multifunctional type-3 copper-containing metalloenzyme, is widely distributed in nature.[1,2] It has a diverse roles in agriculture, the cosmetic industry and medicine.[3] The enzyme catalyzes two reactions in the melanin biosynthesis pathway involving molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (cresolase/monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase/diphenolase activity). The produced quinones are highly reactive and can further oxidized spontaneously to form brown pigments of high molecular weight, namely melanin, which are responsible for mammalian skin and hair color.[3] However, recently studies showed that the abnormal accumulation of pigmentation causes the quality loss of vegetables and fruits and various dermatological disorders, such as hyperpigmentation, freckles, melasma, ephelide, and senile lentigines.[4] Furthermore, the melanin pigments are also found in the mammalian brain where tyrosinase plays a key role in the synthesis of neuromelanin and is linked to Parkinson's and other neurodegenerative diseases.[4,5] Therefore, tyrosinase inhibitors should be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation and also important in cosmetics and food industry.[6—8]

So far, a large number of potential tyrosinase inhibitors have been described from natural source, such as kojic acid (Fig. 1),[9] corticosteroids,[10] retinoids,[10] arbutin,[10] tropolone (Fig. 1).[11,12] Unfortunately, only few of these reported compounds are used in practice 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 by laboratory synthesis.[13]

In previous reports, some functionalized 4-alkoxybenzaldehyde derivatives have been presented as potent tyrosinase inhibitors, and further study suggested that the electron-donating groups at C-4 are necessary for the actions.[14,15] Recently, our investigation[16—19] demonstrated that thiosemicarbzide derivatives exhibited profound tyrosinase inhibitory activities because of their strong interaction with the dicopper ions active center of tyrosinase. For example, phenylmethylenethiosemicarbazone (Fig. 1) showed a potent tyrosianse inhibitory activity with the IC_{50} value of 1.93 μM.[19] In addition, it was worth noting that, on the basis of the reported crystallographic structure of tyrosinase, Khatib *et al.* designed and synthesized a series of 3-(2,4-dihydroxyphenyl)propionic acid esters as tyrosinase inhibitors, and the results showed that these compounds have significant activities against tyrosinase.[4] This finding emphasized the possibility that a hydrophobic moiety may also contribute to the inhibition.

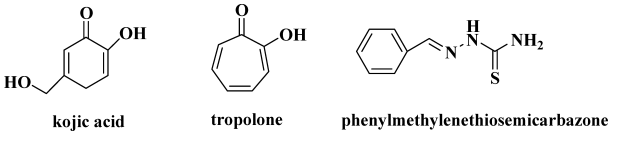

Fig. 1. Structures of Some Known Tyrosinase Inhibitors

Taking advantage of above information, we speculated that the introduction of a proper hydrophobic subunit to position-4 of phenylmethylenethiosemicarbazone, may interact with the hydrophobic enzyme pocket and the dicopper ions active center of tyrosinase, leading to increased inhibitor-enzyme binding affinity, and thus to an improved compound activity. Therefore, in the present study, a series of 4-*O*-substituted phenylmethylenethiosemicarbazones were designed, synthesized and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were evaluated. Moreover, the structure–activity relationships of these compounds were also discussed. We hope that these findings can lead to the discovery of food preservatives and therapeutically potent agents against clinically dermatological disorders including hyperpigmentation as well as skin melanoma and offer the efficient informations for further designing the tyrosinase inhibitor.

Results and Discussion

The synthesis of compounds **2a**—**g** had been described detailedly in our previous report.[20] The procedure for the preparation of compounds **2h**—**t** and **3a**—**t** was outlined in Chart 1. Briefly, compounds **2h**—**t** were synthesized by the reacting 4-hydroxybenzaldehyde with 3-(bromomethyl)pyridine in anhydrous *N*,*N*-dimethylformamide (DMF)/NaH or the corresponding alkyl bromide in the presence of anhy-

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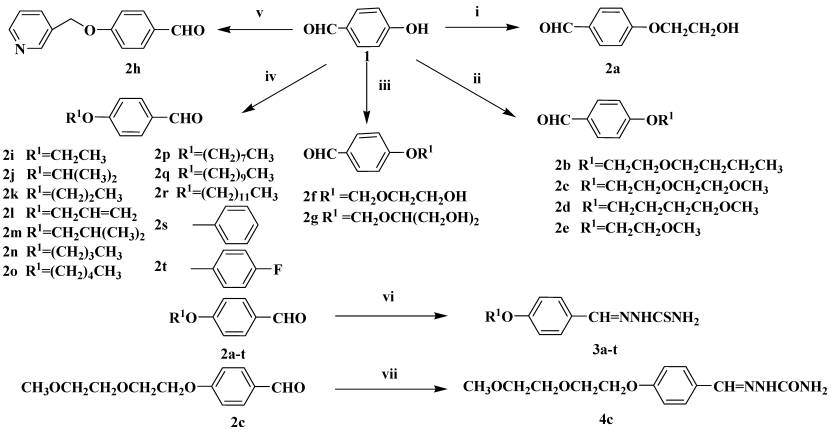

Reagents and conditions: (i) 2-chloroethanol, 1,4-dioxane, NaOH, H_2O, reflux, 2 h; (ii) 2-butoxyethanol or 2-(2-methoxyethoxy)ethanol or 4-methoxybutan-1-ol or 2-methoxyethanol, NaOH, TsCl, 0 °C, 5 h; K_2CO_3, THF, reflux, 16 h; (iii) 2-oxo-1,4-butanediol diacetate ester or 2-[(propionyloxy)methyl-hydrogen]-1,3-propane-diol diacetate, toluene, 4-methylbenzenesulfonic acid, 100 °C, 0.5 h, then reflux, 10 h; methanol, sodium methoxide, rt, 1 h. epichlorohydrin, 1,4-dioxane, NaOH, H_2O, reflux, 2 h; (iv) the corresponding alkyl bromide, K_2CO_3, acetone, reflux, 2—5 h; (v) 3-(bromomethyl)pyridine, NaH, DMF, rt, 2 h; (vi) ethanol, thiosemicarbazide, acetic acid, reflux, 5—24 h; (vii) ethanol, semicarbazide, acetic acid, reflux, 6 h.

Chart 1. Synthesis of Compounds **2a**—**t**, **3a**—**t** and **4c**

drous acetone/K_2CO_3. Subsequently, the reaction of obtained compounds **2a**—**t** with thiosemicarbazide and semicarbazide to give the target compounds **3a**—**t** and **4c**, respectively. The structures of the synthesized compounds were determined by different spectroscopic techniques, like ^{1}H-NMR and EI-MS, and purities were confirmed by elemental analysis.[21)]

The inhibition of our synthetic 4-*O*-substituted phenylmethylenethiosemicarbazones on the diphenolase activity of mushroom tyrosinase was investigated using the usual procedure[22)] and compared with tropolone,[23)] one of the best tyrosinase inhibitors known so far. The IC_{50} values of all obtained compounds were summarized in Table 1. As predicted, a fair number of compounds were found to have more potent tyrosinase inhibitiory activity than phenylmethylenethiosemicarbazone (IC_{50}=1.93 μM). Interestingly, the IC_{50} values of compounds **3a**—**g**, **3j** and **3s** were of the same magnitude as tropolone (IC_{50}=0.42 μM). Especially, compound **3c** bearing a $-(CH_2)_2O(CH_2)_2OCH_3$ group was found to be the most potent inhibitor with the IC_{50} value of 0.34 μM. However, changing the number of oxygen atom and the length of alkyl part decreased the inhibitory activity on different degree. This might be due to the introduction of other substitution to impede the interaction between this molecule and the enzyme. The results suggested that tyrosinase inhibitory activity associated with the configuration of the substitution at the 4-position of benzene, and in the prensent investigation, $-(CH_2)_2O(CH_2)_2OCH_3$ group represented the most optimal structure for these compounds to exhibit remarkable tyrosinase inhibitory effects.

In addition, compounds **3i** and **3k** exhibited the potent tyrosinase inhibitiory activities with IC_{50} values of 1.92 and 3.03 μM, respectively. Elongation of alkyl chain as in compounds **3n** (IC_{50}=1.31 μM) and **3o** (IC_{50}=1.09 μM) produced slight increase of tyrosinase inhibition, while further elongation of alkyl chain to give compounds **3p**, **3q** and **3r**, which showed no tyrosinase inhibitory effect at the concentration of 200 μM. These results implied that the length of alkyl chain contained in the chain attached on position-4 of phenylmethylenethiosemicarbazone might be played a very vital role in determining their inhibitory activities, and the excessive elongation of the alkyl chain retarded the binding of inhibitor and the active site of tyrosinase, leading to a decrease of tyrosinase inhibitiory activity. Moreover, compound **3j** (IC_{50}=0.90 μM) displayed 2.1 times higher tyrosinase inhibitory activity than compound **3i** (IC_{50}=1.92 μM). Compound **3m** (IC_{50}=2.21 μM) showed more potential tyrosinase inhibitory activity than compound **3k** (IC_{50}=3.03 μM). The results suggested that the introduction of secondary alkyl group in the side chain might be beneficial to tyrosinase inhibitory activity, which supported the previous studies reported by Nihei *et al.*[24)] Notably, compound **3l** (IC_{50}=1.29 μM) bearing the $-CH_2CH=CH_2$ group in the side chain exhibited more potential tyrosinase inhibitory activity than **3m**, indicating that the introduction of the $-CH_2CH=CH_2$ group was preferable to $-CH(CH_3)_2$ group in inhibition of tyrosinase. The reason might be that the $-CH_2CH=CH_2$ moiety formed the certain interaction with the hydrophobic protein pocket surrounding the binuclear copper active site.

To further expand the structure–activity relationships, com-

Table 1. Inhibitory Effects on Mushroom Tyrosinase of 4-*O*-Substituted Phenylmethylenethiosemicarbazones **3a**—**t** and Analogue **4c**, Thiosemicarbazide, 4-Hydroxybenzaldehyde and Tropolone

Compound	IC_{50} (μmol/l)[a)]	Compound	IC_{50} (μmol/l)[a)]
3a	0.53±0.092	**3m**	2.21±0.281
3b	0.92±0.109	**3n**	1.31±0.132
3c	0.34±0.054	**3o**	1.09±0.165
3d	0.41±0.065	**3p**	>200
3e	0.75±0.063	**3q**	>200
3f	0.37±0.082	**3r**	>200
3g	0.94±0.133	**3s**	0.98±0.059
3h	3.27±0.335	**3t**	1.46±0.236
3i	1.92±0.159	**4c**	>200
3j	0.90±0.098	Thiosemicarbazide[b)]	>2000
3k	3.03±0.168	4-Hydroxybenzaldehyde[c)]	1220±52
3l	1.29±0.112	Tropolone[d)]	0.42±0.051

a) Assay performed using mushroom tyrosinase. Values are means of three different experiments. IC_{50}=mean±S.E.M., S.E.M.: standard error of mean. *b*) The concentration of 2.00 (mM) corresponding to inhibition percentage, determined in this work, is 39.9±2.5%. *c*) Values in the literature[8)] is 1.20 mM. *d*) Values in the literature is 0.40 μM.[23)]

pounds **3s** and **3t** bearing a benzoxyl group were examined for the tyrosinase inhibitory activity. The results demonstrated that compounds **3s** and **3t** also exhibited significant inhibitory activities with are IC_{50} values of 0.98 and 1.46 μM, respectively, which are more potent active than 2-(phenylmethylene)-thiosemicarbazone (IC_{50}=1.93 μM). However, introducing a pyridine methyleneoxyl group to position-4 of 2-(phenylmethylene)-thiosemicarbazone as in compound **3h** (IC_{50}= 3.27 μM) led to a loss of tyrosinase inhibitory activity. It was suggested that the nature of aromatic ring might influence the tyrosinase inhibitory potency, and in the present investigation, benzoxyl group was much preferable to pyridine methyleneoxyl group in inhibition of tyrosinase. Moreover, when the thiosemicarbazide moiety of the most active compound **3c** was replaced by the semicarbazide group, the obtain compound **4c** was inactive at the concentration of 200 μM. The results showed that the sulfur atom of thiosemicarbazide moiety was absolutely necessarily for determining the tyrosinase inhibitory activity of these compounds, which futher supported our previous report.[18)]

Conclusion

In this study, a series of 4-*O*-substituted phenylmethylenethiosemicarbazones were designed, synthesized and evaluated as mushroom tyrosinase inhibitors. The results demonstrated that most of compounds had potent tyrosinase inhibitory activities. Interestingly, the IC_{50} values of compounds **3a**—**g**, **3j** and **3s** were of the same magnitude as tropolone. Especially, compound **3c** bearing a $-(CH_2)_2O(CH_2)_2OCH_3$ group was found to be the most potent inhibitor with the IC_{50} value of 0.34 μM. Structure–activity relationships (SARs) analysis showed that: (1) Tyrosinase inhibitory activity associated with the configuration of the substitution at the 4-position of benzene. (2) The length of alkyl chain contained in the chain attached on position-4 of phenylmethylenethiosemicarbazone might be played a very vital role in determing the inhibitory activity. (3) The introduction of secondary alkyl group in the side chain might be beneficial to tyrosinase inhibitory activity. (4) The nature of aromatic ring might influence the tyrosinase inhibitory potency. (5) The sulfur atom of thiosemicarbazide moiety was absolutely necessary for determining the tyrosinase inhibitory activity. All these data suggested that these molecules might be utilized for the development of new candidate for the treatment of dermatological disorders. Further modification based on the current obtained SARs and evaluation of these potent compounds using a human melanoma cell line would be continued in our laboratory, and the research results will be reported in due course.

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21) General procedures for the synthesis of compounds **3a**—**t** and **4c**: The appropriate compounds **2a**—**t** (1 mmol) were dissolved in anhydrous ethanol (15 ml), thiosemicarbazide or semicarbazide (1 mmol) was added to the above solution. The reaction mixture was refluxed for 5—24 h and cooled to room temperature. The precipitate was filtered, washed with ethyl ether, and recrystallized from 95% alcohol to give compounds **3a**—**t** and **4c** in 58—95% yields. Compound **3c** (1-(1-(4-(2-(2-methoxyethoxyl)ethoxyl))benzyliene)thiosemicarbazide): light orange powder, yield 93%, mp 141—142 °C. IR (KBr, cm^{-1}) *v*: 3394, 3293, 3158, 3040, 2862, 2851, 1604, 1537, 1259, 1174, 1131, 931, 828, 790; ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 11.28 (s, 1H, NH), 8.08 (s, 1H, NH), 7.97 (s, 1H, –CH=N–), 7.89 (s, 1H, NH), 7.72 (d, J= 9.0 Hz, 2H, Ar-H), 6.97 (d, J=9.0 Hz, 2H, ArH), 4.14 (t, J=5.0 Hz, 2H, $-CH_2-$), 3.75 (t, J=4.5Hz, 2H, $-CH_2-$), 3.59(t, J=4.5 Hz, 2H, $-CH_2-$), 3.43 (t, J=3.9 Hz, 2H, $-CH_2-$), 3.23 (s, 3H, $-OCH_3$); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ: 178.6, 161.4, 144.4, 129.5, 126.2, 115.4, 72.4, 71.2, 70.0, 67.9, 59.5; ESI-MS: *m*/*z* 320 (M+Na, 100). *Anal.* Calcd: C, 52.51; H, 6.44; N, 14.13. Found: C, 52.54; H, 6.67; N, 14.19.
22) Tyrosinase inhibition assays were performed as follows: 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 dimethyl sulfoxide (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) was first pre-incubated with the compounds, in 50 mM phosphate buffer (pH 6.8), for 10 min at 25 °C. Then the 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 of the DOPAchrome for 1 min. IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose–response curves. Here, 4-hydroxybenzaldehyde and tropolone were used as the reference inhibitors.
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