Bioorganic & Medicinal Chemistry 18 (2010) 4042–4048

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

New potent inhibitors of tyrosinase: Novel clues to binding of 1,3,4-thiadiazole-2(3*H*)-thiones, 1,3,4-oxadiazole-2(3*H*)-thiones, 4-amino-1,2,4-triazole-5(4*H*)-thiones, and substituted hydrazides to the dicopper active site

Usman Ghani [a,*], Nisar Ullah [b]

[a] Department of Medical Biochemistry, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia
[b] Chemistry Department, King Fahad University of Petroleum and Minerals, Dhahran 31261, Saudi Arabia

ARTICLE INFO

Article history:
Received 24 February 2010
Revised 3 April 2010
Accepted 6 April 2010
Available online 13 April 2010

Keywords:
Tyrosinase inhibitor
1,2,4-Thiadiazole
1,3,4-Oxadiazole
4-Amino-1,2,4-triazole
Substituted hydrazides
Dicopper center
Mono-dentate binding

ABSTRACT

A series of 1,3,4-thiadiazole-2(3*H*)-thiones, 1,3,4-oxadiazole-2(3*H*)-thiones, 4-amino-1,2,4-triazole-5(4*H*)-thiones, and substituted hydrazides were tailored and synthesized as new potent inhibitors of tyrosinase. The rationale for inhibitor design was based on the active site structural evidence from the crystal structures of bacterial tyrosinase and potato catechol oxidase enzymes. Kinetic and active site binding studies suggested mono-dentate binding of thiadiazole, oxadiazole, and triazole rings to the active site dicopper center of tyrosinase including hydrophobicity contributing to the potent inhibition. Kinetic plots showed mixed-type of inhibition by all 25 compounds. Substitutions at C3 of the triazole ring and C5 of the thiadiazole/oxadiazole rings were found to be playing a major role in the high binding affinity to tyrosinase. The current work may help develop new potent tyrosinase inhibitors against hyperpigmentation including potential insecticides.

1. Introduction

Tyrosinase (monophenol, *o*-diphenol:oxygen oxidoreductase; EC 1.14.18.1) is a copper-containing enzyme responsible for biosynthesis of melanin pigment in skin, hair, and eyes.[1,2] It catalyzes two reactions in the melanin biosynthetic pathway (Raper–Mason pathway): hydroxylation and oxidation of monophenols to *o*-quinones (monophenolase activity), and oxidation of *o*-diphenols to *o*-quinones (*o*-diphenolase activity). The enzyme is widely distributed in mammals, plants, insects, fungi, and bacteria.[3,4]

The initial steps of the Raper–Mason pathway have been targets in the course of searching for inhibitors of tyrosinase.[5] Certain diseases or clinical conditions related to hyperpigmentation such as flecks and skin defects are associated with the excessive production of melanin.[6] Tyrosinase has also been linked to Parkinson's and other neurodegenerative diseases.[7,8] Therefore, identification of tyrosinase inhibitors has promising potential in the treatment of such ailments.[5] Tyrosinase inhibitors have become increasingly important for cosmetic and medicinal products primarily in relation to hyperpigmentation of skin. Topical preparations containing tyrosinase inhibitors are used for the treatment of localized hyperpigmentation in humans such as lentigo, nevus, ephelis, post-inflammatory state, and melanoma of pregnancy.[9]

Inhibition of tyrosinase is equally important commercially. In most fruits and vegetables, the enzyme is responsible for undesired browning that takes place during senescence or damage during post-harvest handling, leading to faster degradation and shorter shelf life.[10] Therefore, inhibition of tyrosinase is desirable in order to control browning and reduce economic losses. In insects, the molting process and wound healing by melanin are critical to their survival.[11] Furthermore, quinonoid intermediates generated by tyrosinase serve as defense molecules for insects.[12] These processes provide potential targets for developing safer and effective tyrosinase inhibitors as insecticides and ultimately for insect control.

In view of exploring new, potent and safer inhibitors of tyrosinase, we synthesized a series of 1,3,4-thiadiazole-2(3*H*)-thiones, 1,3,4-oxadiazole-2(3*H*)-thiones, 4-amino-1,2,4-triazole-5(4*H*)-thiones, and substituted hydrazides. Some of these compounds have been reported as urease inhibitors[13] including analogues of 1,3,4-oxadiazole-2(3*H*)-thiones which exhibit pyrophosphatase and phosphodiesterase I inhibitory activities as reported elsewhere.[14] There are no reports of these compounds on tyrosinase inhibition. Tyrosinase inhibitors from natural or synthetic origins have been studied widely[15–24] including substrate analogs[25,26] and inhibitors

* Corresponding author. Tel.: +966 1 4690140; fax: +966 1 4672575.
E-mail addresses: ughani@ksu.edu.sa, ughani@gmx.net (U. Ghani).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.04.021

different from substrate such as thiones[27] and triazoles.[28–31] An extensive literature search indicated that the compounds reported in this study are new inhibitors of tyrosinase, and some of them are even more potent than the standard inhibitors such as kojic acid[32] and L-mimosine.[33] Our kinetic and structure–activity relationship studies revealed novel clues to the identification, active site binding and mechanism of action of the pharmacophores present in these inhibitors.

No crystal or NMR structure of mushroom tyrosinase is yet available, however, there is a significant amount of chemical and spectroscopic data available that gives important information on the geometric and electronic structure of mushroom tyrosinase active site.[34] Crystal structure of the enzymes closely related to tyrosinase such as catechol oxidase from potato[35] and the odg domain of the respiratory pigment hemocyanin from *Octopus dofleini*[36] have been solved. Although crystallization of tyrosinases presents serious problems partially due to their high hydrophobicity, Matoba et al.[37] were able to successfully determine the first high resolution crystal structure of tyrosinase from *Streptomyces castaneoglobisporus* bacteria (bacterial tyrosinase).

These enzymes including mushroom tyrosinase contain a type-3 copper center in their active site, and their overall structures along with the active site geometries are very similar to each other. Our rationale for inhibitor design and synthesis took vantage of the similarity of the active site structures of the bacterial tyrosinase and catechol oxidase. Both of the enzyme structures provide important information on the chemistry of active site residues and rendered us a basic framework for designing appropriate pharmacophores for these compounds. Our kinetic and binding studies convincingly showed that 1,3,4-thiadiazole-2(3*H*)-thione, 1,3,4-oxadiazole-2(3*H*)-thione, and 4-amino-1,2,4-triazole-5(4*H*)-thione rings bind to the dicopper center of tyrosinase in a mono-dentate fashion with the major contribution of hydrophobic interactions with the active site.

We present for the first time, a rationale for binding of these inhibitors to tyrosinase active site that has not been explored before. It would certainly highlight new insights into designing and optimizing better tyrosinase inhibitors. Despite difficulties in tyrosinase crystallization, our attempts are already in progress to verify our binding proposal by determining crystal structure of the inhibitors in complex with mushroom and bacterial tyrosinases, as no tyrosinase–inhibitor complex structure is available to date.

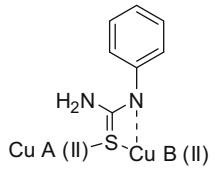
2. Results and discussion

2.1. Rationale for inhibitor design and synthesis

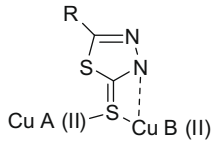
The crystal structure of the bacterial tyrosinase reveals important information about its active site. The core of the enzyme, containing the active site, is formed by a four-helix-bundle where the catalytic dicopper center is located. This center is located at the bottom of a large hydrophobic substrate binding pocket formed by the side chains of hydrophobic amino acids.[37] Other enzyme, structurally and functionally related to tyrosinase, is catechol oxidase–phenylthiourea (PTU) complex.[35] The active site structure of the enzyme is very similar to that of the bacterial tyrosinase[37] even though catechol oxidases show no monooxygenase activity whereas tyrosinases exhibit both monooxygenase and oxidase activities.[35]

The two most important factors responsible for catechol oxidase inhibition by PTU are: (i) coordination of the sulfur atom of PTU with the dicopper active site and (ii) hydrophobic interactions. The sulfur atom of the inhibitor replaces the hydroxo-bridge present in the Cu(II)–Cu(II) active site, and is coordinated to both copper ions. Moreover, the amide nitrogen of the inhibitor weakly interacts with Cu^B making a square-pyramidal coordination sphere (Fig. 1A). The dicopper center of the enzyme is located in the center of a hydrophobic pocket contributed by the side chains of hydrophobic amino acids.

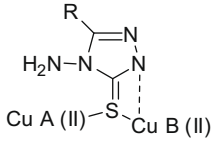
Both of the above studies are promising in terms of providing clues to understand the mechanism of tyrosinase inhibition and in identifying inhibitors that may bind to tyrosinase in a manner similar to that of PTU. We propose mono-dentate binding of 1,3,4-thiadiazole-2(3*H*)-thione, 4-amino-1,2,4-triazole-5(4*H*)-thione, and 1,3,4-oxadiazole-2(3*H*)-thione rings to the dicopper center of tyrosinase. The sulfur atom of the thione moiety of these compounds is proposed to replace the hydroxo-bridge in Cu(II)–Cu(II) center, and is coordinated to both copper ions whereas the adjacent thioamidic nitrogen atom is proposed to interact with the Cu^B of the center similar to PTU coordination (Fig. 1B–D). Previous molecular simulation studies have indicated that the binding of phenolic oxygen of tyrosine substrate to Cu^B may be necessary as the first step for catalysis.[38,39] Moreover, hydrophobicity of the inhibitors is likely contributing to the high affinity since most of the chemical groups in these compounds are hydrophobic in nature. We propose that while 1,3,4-thiadiazole-2(3*H*)-thione, 4-amino-1,2,4-triazole-5(4*H*)-thione, and 1,3,4-oxadiazole-2(3*H*)-thione rings coordinate with the dicopper center, other chemical groups bind to the active site



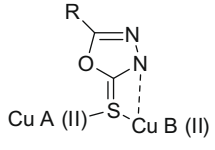
(A)



(B)



(C)



(D)

Figure 1. (A) Binding of PTU to the active site dicopper center of potato catechol oxidase as observed in the crystal structure of the complex (Klabunde et al., 1998). Proposed binding of (B) thiadiazole, (C) triazole, and (D) oxadiazole rings to the active site dicopper center of mushroom tyrosinase.

mainly through hydrophobic interactions along with some non-hydrophobic interactions.

2.2. Kinetic studies

Kinetic studies showed a concentration-dependent inhibition of mushroom tyrosinase by the compounds. Continuous monitoring of the reaction in the presence of the inhibitors showed a marked decrease in the reaction rate, that is, ultimately indicated by a decrease in the final absorbance when compared with controls containing no inhibitor. The potency of inhibition exhibited by these compounds varied depending on the class of compounds and the presence and positions of different substitutions. The general order of inhibition potency was found to be: thiadiazoles > triazoles > oxadiazoles > substituted hydrazides.

The structures and K_i values of the inhibitors are listed in Table 1, and are compared with kojic acid. The most potent were **S1** (K_i = 0.19 μM), **S2** (K_i = 0.49 μM), **S3** (K_i = 0.53 μM), **N8** (K_i = 1.01 μM), **O10** (K_i = 1.35 μM), and **O11** (K_i = 1.77 μM). The K_i values of thiadiazoles, triazoles, oxadiazoles, and substituted hydrazides lie in the range of 0.19–5.2 μM, 1.01–2.4 μM, 1.35–69.4 μM, and 49–177.2 μM, respectively.

Inhibition kinetics was analyzed by Dixon plot[40] to determine the type of inhibition and K_i value. Slopes obtained from the plots for uninhibited enzyme and with different concentrations of the inhibitors were consistent with the characteristic patterns of mixed-type inhibition, similar to kojic acid which exerts a mixed-type inhibitory effect on the *o*-diphenolase activity of mushroom tyrosinase.[41,15] Representative Dixon plots for inhibitors from each class are shown in Figure 2. A comparison of the K_i values of the compounds with that of urease inhibitors[13] revealed that they possess much higher affinity to tyrosinase than urease. It is likely that this high potency renders them more selective toward tyrosinase than urease.

2.3. Inhibitor–dicopper center binding

Binding studies were performed to determine whether the inhibitors were able to interact specifically with the dicopper active site of tyrosinase. **O20** (10 μM) inhibited the *o*-diphenolase activity by indicating ΔA_{475nm} of 0.074/min when compared with control containing no inhibitor (0.165/min). As shown in Figure 3A, a concentration of 25 μM $CuSO_4$ was required to achieve 70% reversal of **O20**-inhibited tyrosinase activity. The activity did not fully recover because increasing concentrations of $CuSO_4$ itself inhibited the enzyme activity (Fig. 3B), and probably due to some remaining affinity of the inhibitor to the active site. Since only 25 μM $CuSO_4$ was required to recover the **O20**-inhibited tyrosinase activity, it is proposed that the **O20**–tyrosinase complex is dissociable, and **O20** is a reversible inhibitor.

The effect of **O10** (K_i = 1.35 μM) on the activity reversal of tyrosinase was also studied since this inhibitor was more potent than **O20**. As shown in Figure 3C, a higher concentration of $CuSO_4$ (~300 μM) was required to achieve maximum recovery (~37%) of **O10**-inhibited tyrosinase activity. This can be explained by the fact that **O10** carries higher affinity for the active site than **O20**, therefore, lesser effect of $CuSO_4$ was observed in recovering the **O10**-inhibited tyrosinase activity. It is implied that higher concentration of $CuSO_4$ was required to regain partial tyrosinase activity if bound to more potent inhibitors. The studies are convincing to conclude that the compounds specifically interact with the dicopper active site of tyrosinase. Therefore, it is unlikely that a simple chelation effect might be responsible for the inhibitory action of these compounds.

Table 1
Structures of thiadiazole, triazole, oxadiazole, and substituted hydrazide inhibitors with K_i values expressed as SEM (standard error of mean)

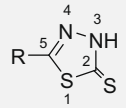
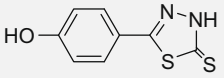
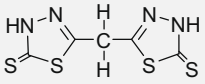
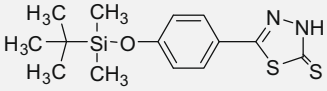
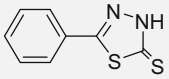
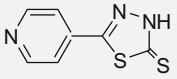
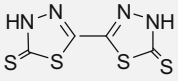
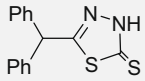
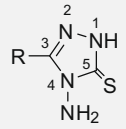
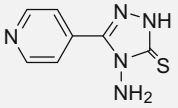
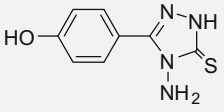
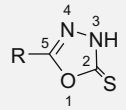
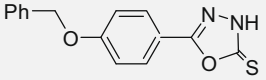
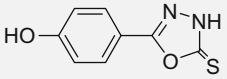
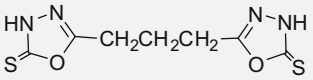
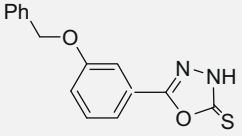
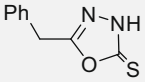
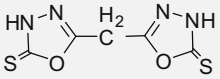
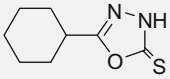
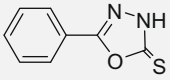
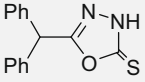
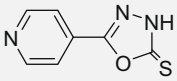
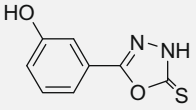
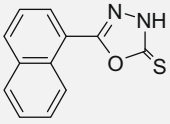
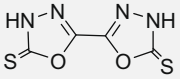
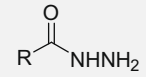
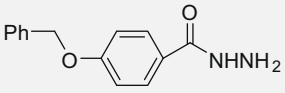
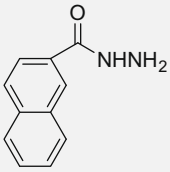
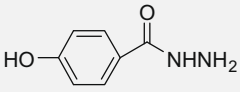
No.	Structure	K_i (μM ± SEM)
	 1,3,4-Thiadiazole-2(3*H*)-thione	
S1		0.19 ± 0.002
S2		0.49 ± 0.005
S3		0.53 ± 0.008
S4		1.31 ± 0.018
S5		2.47 ± 0.038
S6		2.90 ± 0.046
S7		5.20 ± 0.117
	 4-Amino-1,2,4-triazole-5(4*H*)-thione	
N8		1.01 ± 0.01
N9		2.40 ± 0.068
	 1,3,4-Oxadiazole-2(3*H*)-thione	
O10		1.35 ± 0.10
O11		1.77 ± 0.038
O12		1.90 ± 0.049
O13		3.64 ± 0.23

Table 1 (*continued*)

No.	Structure	K_i (μM ± SEM)
O14		3.67 ± 0.073
O15		4.42 ± 0.078
O16		4.94 ± 0.11
O17		6.47 ± 0.24
O18		6.76 ± 0.15
O19		14.2 ± 0.86
O20		18.5 ± 0.36
O21		33.6 ± 1.31
O22		69.4 ± 0.48
	 Substituted hydrazides	
H23		49.0 ± 0.96
H24		99.6 ± 0.41
H25		177.2 ± 15.2
	Kojic acid	22.3 ± 0.71

2.4. Structure–activity relationship

2.4.1. 1,3,4-Thiadiazole-2(3*H*)-thiones

Compounds in this class were found to be the most potent inhibitors. Among them the most potent was **S1** containing a 5-(4-hydroxyphenyl) substitution (K_i = 0.19 μM). The inhibitory activity of the compound decreased more than sixfold when the 4-hydroxyl group on the phenyl ring was removed converting it to **S4** containing a 5-hydroxyphenyl group (K_i = 1.31 μM) reflecting the importance of the hydroxyl group at position 4 and the phenyl ring in enhancing the inhibitory activity of **S1**. High affinity of **S1** is most likely due to hydrogen-bonding interactions with one of the active site residues.

Two more substitutions at the same position of the phenyl ring with hydrophobic groups yielded better inhibitors (**S2**; K_i = 0.49 μM and **S3**; K_i = 0.53 μM). We propose this to be mainly due to hydrophobic interactions with the active site. However, **S5** bearing a relatively polar group (4-pyridyl) showed a significant decrease in the activity (K_i = 2.47 μM) when compared with other inhibitors in the same series such as **S2** and **S3**. The K_i values of **S5** and **S6** are comparable since they are structurally similar to each other (Table 1). A relatively moderate inhibitory activity of **S7** (K_i = 5.20 μM) was observed containing a 5-diphenylmethyl group possibly explaining some role of the bulkiness of the two phenyl rings which may poorly fit in the substrate binding pocket.

2.4.2. 4-Amino-1,2,4-triazole-5(4*H*)-thiones

The inhibitory activities of triazoles were comparable with that of some inhibitors of the thiadiazole and oxadiazole series. Although the substitutions in **S1** and **N9** are identical (4-hydroxyphenyl), the difference in the main moieties makes **S1** remarkably 12-fold more potent than its counterpart **N9**. It is assumed that generally the thiadiazole ring can better fit into the dicopper center of enzyme than that of triazole. However, this assumption cannot be applied exclusively to the main moieties due to the observation that substituted groups also importantly contribute to the potency as verified by comparing the structures and K_i values of **S5** (2.47 μM) and **N8** (1.01 μM).

2.4.3. 1,3,4-Oxadiazole-2(3*H*)-thiones

In this series, 10 different derivatives were tested for inhibitory activity. **O10** bearing a 4-benzyloxyphenyl group on the oxadiazole ring was found to be the most potent inhibitor (K_i = 1.35 μM). Comparison of this inhibitor with its positional isomer **O13** (benzyloxy group of the phenyl ring at position 3) showed a threefold decrease in the activity. It revealed that the isomers with benzyloxy group at position 4 of the phenyl ring are more preferred by the active site for binding. **O11** containing a 4-hydroxyphenyl group at position 5 of the oxadiazole ring was the second most potent inhibitor in this series.

It is interesting to note that both the absence of a hydroxyl group from phenyl ring (**O17**) and the presence of a cyclohexyl ring as a substituent at 5-position (**O16**) decreased the inhibitory activity several folds. This is similar to what was observed earlier in the thiadiazole and triazole series that the presence of a 5-(4-hydroxy) phenyl group exhibits potent inhibitory activity against tyrosinase. However, a comparison of structures indicated that the potency varied depending on the class of compounds and the type of substitutions.

Very interestingly, **O20** (K_i = 18.5 μM), a positional isomer of **O11** due to a hydroxyl group, exhibited 10-fold less inhibition than that of **O11** (K_i = 1.77 μM), which further confirmed the importance of the hydroxyl group at position 4 of phenyl substituent in exhibiting potent inhibition. We attribute this potency to hydrogen-bonding interaction(s) with specific active site amino acids. Presence of hydroxyl group renders an advantage of optimally approaching those amino acids in order to form hydrogen bonding.

A comparison of **O12** (K_i = 1.90 μM), **O15** (K_i = 4.42 μM), and **O22** (K_i = 69.4 μM) suggested that the inhibitory activity of these compounds varied according to the number of the carbon atoms connecting the two oxadiazole rings. The activity of **O12** decreased twofold when the carbon chain is shortened by two atoms giving rise to **O15**. A marked decrease in the activity was observed when the two oxadiazole rings were directly bonded to each other (as in **O22**). An interesting clue can be derived from these observations that inhibitor size is also important in the binding; longer molecules may bind to the active site better than the shorter ones. Since the dicopper center is located at the bottom of a large substrate binding pocket,[25] it is assumed that longer molecules are at ease

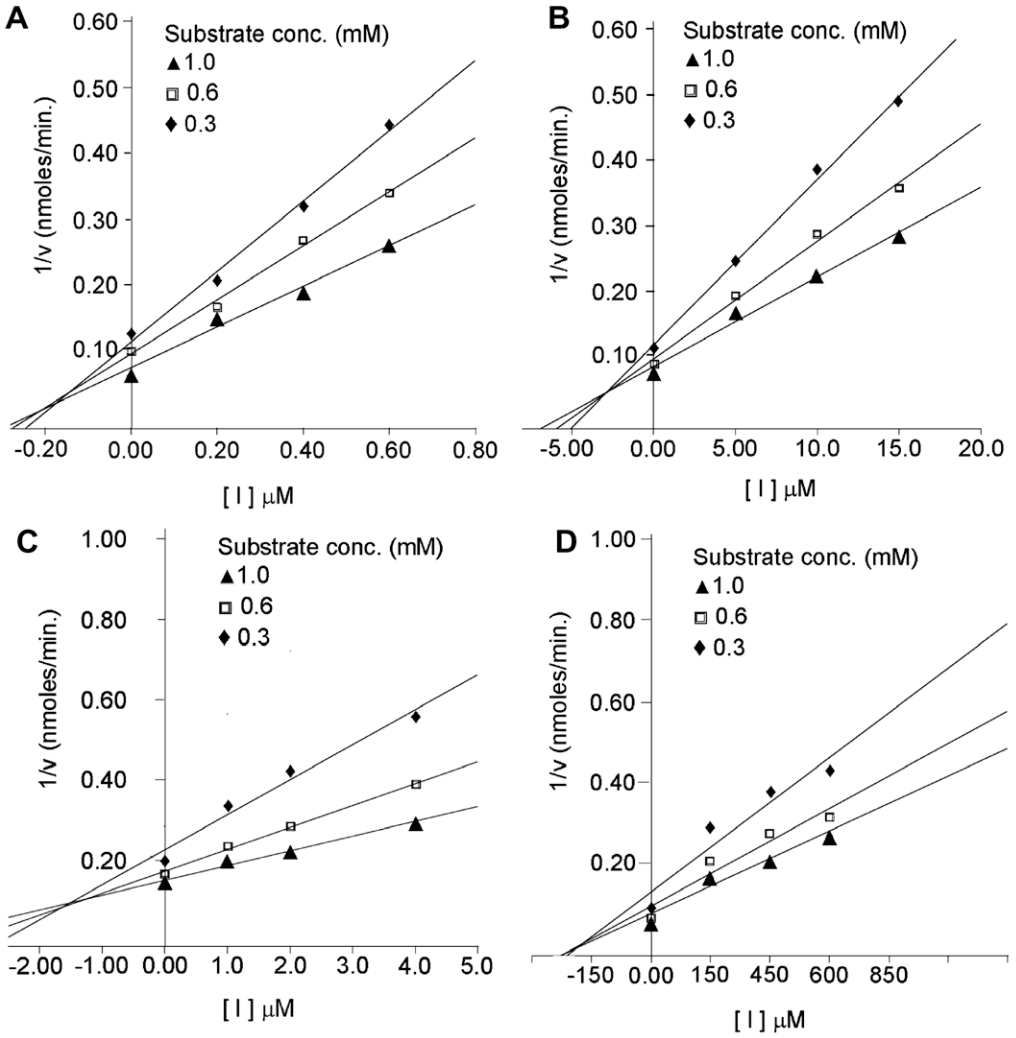


Figure 2. Representative Dixon plots for (A) thidiazole **S1**, (B) triazole **N9**, (C) oxadiazole **O10**, and (D) substituted hydrazide **H23** inhibitors.

in approaching and interacting with the dicopper center by taking advantage of the corresponding size and dimensions of the substrate binding pocket. Compounds with diphenylmethyl (**O18**) or 1-naphthyl (**O21**) substituents exhibited weaker inhibitory activity that may be due to the bulkiness of the structures as discussed earlier for similar inhibitors.

2.4.4. Substituted hydrazides

The structural dissimilarity of the substituted hydrazides to thiadiazoles, triazoles, and oxadiazoles may indicate a different binding mode of these inhibitors to tyrosinase active site. Comparison of K_i values with other classes of inhibitors studied in this work showed that these compounds were moderate inhibitors of tyrosinase. **H23**, **H24**, and **H25** contain substitutions identical to the ones present in thiadiazoles, triazoles, and oxadiazoles. It was observed that the inhibitory potency greatly varied according to the type of substitutions. However, the hydrazide moiety should have an important role in the inhibitory activity.[42,43]

3. Conclusions

The current work revealed novel clues to mono-dentate binding of the inhibitors to tyrosinase active site along with hydrophobicity yielding potent inhibition. The work will contribute to further understanding of the mechanism of tyrosinase inhibition and developing effective insecticides and drugs against hyperpigmentation. Further studies are underway to determine crystal structure of the inhibitors in complex with mushroom and bacterial tyrosinases.

4. Experimental

4.1. Chemical synthesis

Synthesis of 1,3,4-oxadiazole-2(3*H*)-thiones was accomplished by condensing the appropriate ethyl ester with hydrazine to generate the corresponding hydrazides, which in turn were heated with carbon disulfide in ethanolic KOH.[44] The thiones obtained were reacted with hydrazine in ethanol to produce 1,2,4-triazole-5(4*H*)-thiones.[45] 1,3,4-Thiadiazole-2(3*H*)-thiones were synthesized by exposing hydrazides to carbon disulfide, followed by acid-induced cyclization in chloroform[46] as shown in Scheme 1.

Carbon–hydrogen–nitrogen ratio (CHN), melting points (Büchi 535), mass spectrometry (MAT-312/JEOL JMS-HX 110), UV spectra (Hitachi U-3200), infrared spectrometry (IR; JASCO A-302), proton nuclear magnetic resonance, and carbon 13 nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR; Brücker AM-300, AM-400/AMX500) were used to determine the structures of the compounds, and their chemical shifts were recorded in ppm.

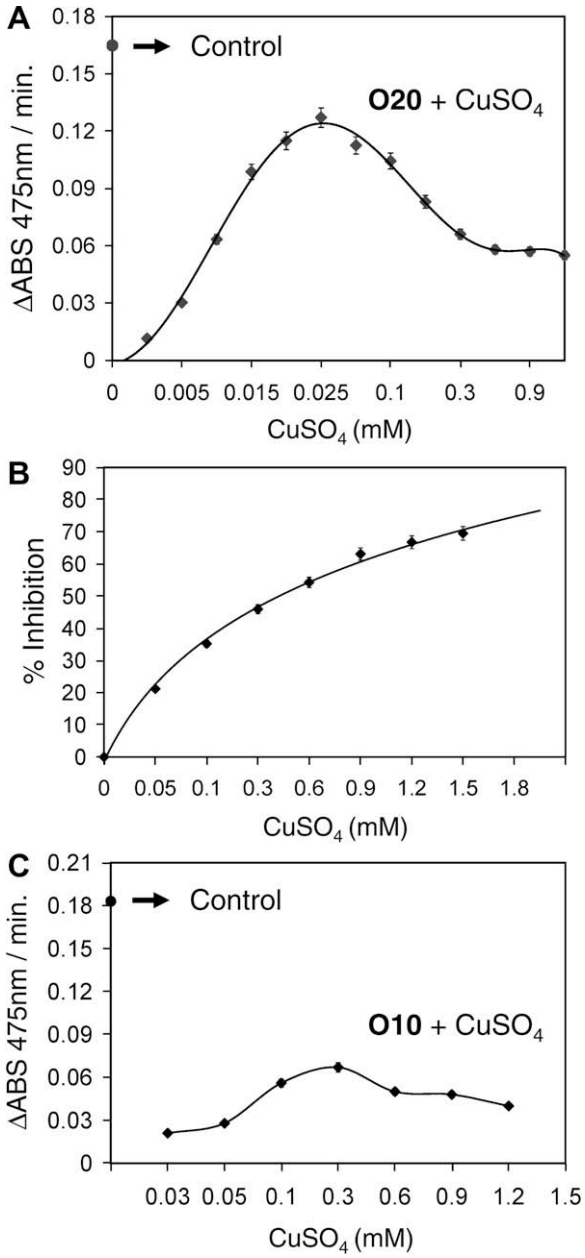
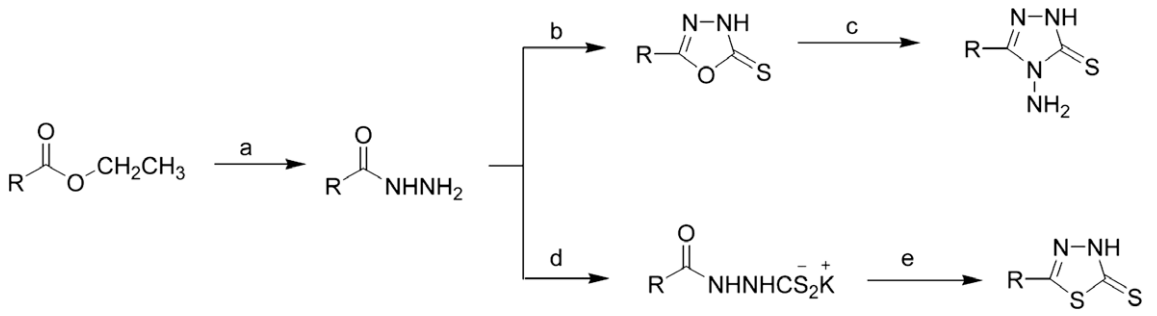


Figure 3. Reversal of the *o*-diphenolase activity of (A) **O20**-inhibited and (B) **O10**-inhibited tyrosinase by $CuSO_4$. (C) Inhibition of *o*-diphenolase activity of tyrosinase by $CuSO_4$ alone.

S1:[14] Anal. Calcd for $C_8H_6N_2OS_2$: C, 45.69; H, 2.88; N, 13.32. Found: C, 45.65; H, 2.91; N, 13.28. **S2**:[14] Anal. Calcd for $C_5H_4N_4S_4$: C, 24.18; H, 1.62; N, 22.56. Found: C, 24.15; H, 1.65; N, 22.51. **S3**:[14] Anal. Calcd for $C_{14}H_{20}N_2OS_2Si$: C, 51.81; H, 6.21; N, 8.63. Found: C, 51.77; H, 6.24; N, 8.58. **S4**: 215–217 °C; Lit. 47a 215 °C. Anal. Calcd for $C_8H_6N_2S_2$: C, 49.46; H, 3.11; N, 14.42. Found: C, 49.42; H, 3.13; N, 14.38. **S5**: 291–293 °C; Lit. 47b 288–291 °C. Anal. Calcd for $C_7H_5N_3S_2$: C, 43.06; H, 2.58; N, 21.52. Found: C, 43.02; H, 2.61; N, 21.48. **S6**:[13] Anal. Calcd for $C_4H_2N_4S_4$: C, 20.50; H, 0.86; N, 23.91. Found: C, 20.47; H, 0.88; N, 23.88. **S7**:[14] Anal. Calcd for $C_{15}H_{12}N_2S_2$: C, 63.35; H, 4.25; N, 9.85. Found: C, 63.31; H, 4.28; N, 9.81. **N8**: 235–236 °C; Lit. 47c 235.5 °C. Anal. Calcd for $C_7H_7N_5S$: C, 43.51; H, 3.65; N, 36.24. Found: C, 43.47; H, 3.69; N, 36.20. **N9**:[47d] Anal. Calcd for $C_8H_8N_4OS$: C, 46.14; H, 3.87; N, 26.90. Found: C, 46.10; H, 3.91; N, 26.85. **O10**:[47e] Anal. Calcd for $C_{15}H_{12}N_2O_2S$: C, 63.36; H, 4.25; N, 9.85. Found: C, 63.32; H, 4.28; N, 9.81. **O11**: 209–210 °C; Lit. 47f 208 °C. Anal. Calcd for $C_8H_6N_2O_2S$: C, 49.47; H, 3.11; N, 14.42. Found: C, 49.44; H, 3.13; N, 14.39. **O12**:[47g] Anal. Calcd for $C_7H_8N_4O_2S_2$: C, 34.42; H, 3.30; N, 22.93. Found: C, 34.38; H, 3.32; N, 22.90. **O13**:[14] Anal. Calcd for $C_{15}H_{12}N_2O_2S$: C, 63.36; H, 4.25; N, 9.85. Found: C, 63.32; H, 4.28; N, 9.81. **O14**: 129–130 °C; Lit. 47h 130–131 °C. Anal. Calcd for $C_9H_8N_2OS$: C, 56.23; H, 4.19; N, 14.57. Found: C, 56.21; H, 4.23; N, 14.51. **O15**:[14] Anal. Calcd for $C_5H_4N_4O_2S_2$: C, 27.77; H, 1.86; N, 25.91. Found: C, 27.73; H, 1.89; N, 25.88. **O16**: 81–82 °C; Lit. 47i 81 °C. Anal. Calcd for $C_8H_{12}N_2OS$: C, 52.15; H, 6.56; N, 15.20. Found: C, 52.11; H, 6.59; N, 15.16. **O17**: 217–218 °C; Lit. 47f 218 °C. Anal. Calcd for $C_8H_6N_2OS$: C, 53.92; H, 3.39; N, 15.72. Found: C, 53.90; H, 3.42; N, 15.68. **O18**:[14] Anal. Calcd for $C_{15}H_{12}N_2OS$: C, 67.14; H, 4.51; N, 10.44. Found: C, 67.10; H, 4.54; N, 10.40. **O19**: 231–232 °C; Lit. 47f 230 °C. Anal. Calcd for $C_7H_5N_3OS$: C, 46.92; H, 2.81; N, 23.45. Found: C, 46.89; H, 2.84; N, 23.40. **O20**:[14] Anal. Calcd for $C_8H_6N_2O_2S$: C, 49.47; H, 3.11; N, 14.42. Found: C, 49.42; H, 3.14; N, 14.38. **O21**: 220–221 °C; Lit. 47h 217–218 °C. Anal. Calcd for $C_{12}H_8N_2OS$: C, 63.14; H, 3.53; N, 12.27. Found: C, 63.10; H, 3.56; N, 12.23. **O22**:[47h] Anal. Calcd for $C_4H_2N_4O_2S_2$: C, 23.76; H, 1.00; N, 27.71. Found: C, 23.72; H, 1.03; N, 27.67. **H23**: 140–141 °C; Lit. 47j 140 °C. Anal. Calcd for $C_{14}H_{14}N_2O_2$: C, 69.41; H, 5.82; N, 11.56. Found: C, 69.37; H, 5.86; N, 11.54. **H24**: 151–152 °C; Lit. 47j 152 °C. Anal. Calcd for $C_{11}H_{10}N_2O$: C, 70.95; H, 5.41; N, 15.04. Found: 70.90; H, 5.45; N, 15.00. **H25**: 262–263 °C; Lit. 47j 261 °C. Anal. Calcd for $C_7H_8N_2O_2$: C, 55.26; H, 5.30; N, 18.41. Found: C, 55.22; H, 5.33; N, 18.38.

4.2. Tyrosinase inhibition assay

The compounds were tested for *o*-diphenolase inhibitory activity of tyrosinase using L-DOPA (dihydroxyphenylalanine) as substrate in a 96-well microplate format using SpectraMax Plus®



Scheme 1. Synthesis of 1,3,4-oxadiazole-2(3*H*)-thiones, 1,2,4-triazole-5(4*H*)-thiones, and 1,3,4-thiadiazole-2(3*H*)-thiones. Reagents and conditions: (a) $NH_2NH_2{\cdot}H_2O$; (b) CS_2, KOH, EtOH, 80 °C, 12 h; (c) $NH_2NH_2{\cdot}H_2O$, EtOH, 90 °C, 8 h; (d) CS_2, KOH, EtOH; (e) H_2SO_4, $CHCl_3$, 0 °C.

microplate reader (Molecular Devices, CA, USA). Inhibitory activity was determined by the method previously reported.[48] All inhibitors were dissolved in DMSO and its final concentration in the reaction mixture was 3%. Mushroom tyrosinase (28 nM; Sigma Chemical Co., St. Louis, USA) was preincubated with the compounds in 50 mM sodium phosphate buffer (pH 6.8) for 20 min at 25 °C. L-DOPA (final concn 0.5 mM) was added to the mixture and the enzyme reaction was continuously monitored by measuring the change in absorbance at 475 nm. Kojic acid was used as standard inhibitor.

4.3. Determination of K_i values and type of inhibition

The reaction mixture contained 50 mM Na-phosphate buffer (pH 6.8), 28 nM tyrosinase, 3% DMSO, and various concentrations of L-DOPA (0.2–1.0 mM). Tyrosinase was preincubated with different concentrations of inhibitors for 20 min at 25 °C and the reaction was initiated by adding substrate. Formation of DOPAchrome was continuously monitored at 475 nm for 2 min in the microplate reader.

Initial enzyme reaction velocity (μmol/min/mg) was calculated using the linear portion of each curve expressed as ΔA_{475}/min. The rate of the reaction was linear for initial 2 min. K_i values and type of inhibition were calculated by the method of Dixon[28] using Grafit software (version 4.09, Erithacus Software Ltd, Staines, UK). Mean K_i values were expressed using SEM (standard error of mean) obtained from each of four individual experiments.

4.4. Dicopper center binding assay

Binding of the inhibitors to tyrosinase active site was determined by the method described elsewhere.[49] Briefly, tyrosinase was preincubated with the highest concentration of inhibitors required to achieve maximum inhibition of the enzyme. $CuSO_4$ was added to the pre-inhibited tyrosinase in a range of concentrations (0.002−1.0 mM) followed by addition of substrate. The reaction was monitored at 475 nm in the microplate reader. Any increase in absorbance was indicative of the activity recovery of pre-inhibited tyrosinase.

Acknowledgment

The authors are deeply grateful to Ms. Maimoona Rasheed for her technical support and fruitful discussions regarding synthesis of the compounds.

References and notes

1. Lerner, A. B.; Fitzpatrick, T. B. *Physiol. Rev.* **1950**, *30*, 91.
2. Fitzpatrick, T. B.; Miyamoto, M.; Ishikawa, K. *Adv. Biol. Skin* **1967**, *8*, 1.
3. Raper, H. S. *Physiol. Rev.* **1928**, *8*, 245.
4. Mason, H. S. *Annu. Rev. Biochem.* **1965**, *34*, 595.
5. Kubo, I.; Kinst-Hori, I.; Yokokawa, Y. *J. Nat. Prod.* **1994**, *57*, 545.
6. Oetting, W. S. *Pigment Cell Res.* **2000**, *13*, 320.
7. Hazes, B.; Magnus, K. A.; Bonaventura, C.; Bonaventura, J.; Kalk, K. H.; Hol, W. G. *Protein Sci.* **1993**, *2*, 597.
8. Hazes, B.; Magnus, K. A.; Kalk, K. H.; Bonaventura, C.; Hol, W. G. *J. Mol. Biol.* **1996**, *262*, 532.
9. Kubo, I.; Yokokawa, Y.; Kinst-Hori, I. *J. Nat. Prod.* **1995**, *58*, 739.
10. Asanuma, M.; Miyazaki, I.; Ogawa, N. *Neurotox. Res.* **2003**, *5*, 165.
11. Kima, Y. J.; Uyama, H. *Cell. Mol. Life Sci.* **2005**, *62*, 1707.
12. Sugumaran, M.; Nelson, E. *Arch. Insect Biochem. Physiol.* **1998**, *38*, 44.
13. Amtul, Z.; Rasheed, M.; Choudhary, M. I.; Supino, R.; Khan, K. M.; Atta-ur-Rahman *Biochem. Biophys. Res. Commun.* **2004**, *319*, 1053.
14. Khan, K. M.; Fatima, N.; Rasheed, M.; Jalil, S.; Ambreen, N.; Perveen, S.; Choudhary, M. I. *Bioorg. Med. Chem.* **2009**, *17*, 7816.
15. Chang, T. S. *Int. J. Mol. Sci.* **2009**, *10*, 2440.
16. Xie, L. P.; Chen, Q. X.; Huang, H.; Wang, H. Z.; Zhang, R. Q. *Biochemistry* **2003**, *68*, 487.
17. Kim, Y. M.; Yun, J.; Lee, C. K.; Lee, H.; Min, K. R.; Kim, Y. *J. Biol. Chem.* **2002**, *277*, 16340.
18. Jones, K.; Hughes, J.; Hong, M.; Jia, Q.; Orndorff, S. *Pigment Cell Res.* **2002**, *15*, 335.
19. Sabudak, T.; Khan, M. T.; Choudhary, M. I.; Oksuz, S. *Nat. Prod. Res.* **2006**, *20*, 665.
20. Devkota, K. P.; Khan, M. T.; Ranjit, R.; Lannang, A. M.; Samreen; Choudhary, M. I. *Nat. Prod. Res.* **2007**, *21*, 321.
21. Khan, M. T.; Choudhary, M. I.; Khan, K. M.; Rani, M.; Atta-ur-Rahman *Bioorg. Med. Chem.* **2005**, *13*, 3385.
22. Liu, J.; Yi, W.; Wan, Y.; Ma, L.; Song, H. *Bioorg. Med. Chem.* **2008**, *16*, 1096.
23. Wei, Y.; Cao, R.; Chen, Z.; Yu, L.; Ma, L.; Song, H. *Chem. Pharm. Bull.* **2009**, *57*, 1273.
24. Yia, W.; Cao, R.; Wena, H.; Yana, Q.; Zhoua, B.; Wana, Y.; Maa, L.; Song, H. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6490.
25. Canovas, F. G.; Tudela, J.; Madrid, C. M.; Varon, R.; Carmona, F. G.; Lozano, J. A. *Biochim. Biophys. Acta* **1987**, *912*, 417.
26. Tudela, J.; Canovas, F. G.; Varon, R.; Jimenez, M.; Garciacarmona, F.; Lozano, J. A. *Biophys. Chem.* **1988**, *30*, 303.
27. Li, S. B.; Nie, H. L.; Zhang, H. T.; Xue, Y.; Chris, B. W.; Zhu, L. M. *Acta Phys.-Chim. Sin.* **2010**, *26*, 215.
28. Motohashi, N.; Nishikawa, H.; Mori, I. *Chem. Pharm. Bull.* **1991**, *39*, 142.
29. Kim, S.; Jung, S. H.; Cho, C. W. *Arch. Pharmacol. Res.* **2008**, *31*, 1363.
30. Thanigaimalai, P.; Lee, K. C.; Bang, S. C.; Lee, J. H.; Yun, C. Y.; Roh, E.; Hwang, B. Y.; Kim, Y.; Jung, S. H. *Bioorg. Med. Chem.* **2010**, *18*, 1135.
31. Thanigaimalai, P.; Lee, K. C.; Bang, S. C.; Lee, J. H.; Yun, C. Y.; Roh, E.; Hwang, B. Y.; Kim, Y.; Jung, S. H. *Bioorg. Med. Chem.* **2010**, *18*, 1555.
32. Chen, J. S.; Wei, C.; Marshall, M. R. *J. Agric. Food Chem.* **1991**, *39*, 1897.
33. Cabanes, J.; García-Cánovas, F.; Tudela, J.; Lozano, J. A.; García-Carmona, F. *Phytochemistry* **1987**, *26*, 917.
34. Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. *Phytochemistry* **1997**, *45*, 1309.
35. Klabunde, T.; Eicken, C.; Sacchettini, J. C.; Krebs, B. *Nat. Struct. Biol.* **1998**, *5*, 1084 (PDB identifier: 1bt3).
36. Cuff, M. E.; Miller, K. I.; van Holde, K. E.; Hendrickson, W. A. *J. Mol. Biol.* **1998**, *278*, 855.
37. Matoba, Y.; Kumagai, T.; Yamamoto, A.; Yoshitsu, H.; Sugiyama, M. *J. Biol. Chem.* **2006**, *281*, 8981 (PDB identifiers: 1wx5, 1wxc, 1wx3, 2ahk, 2ahl, 1wx2).
38. Siegbahn, P. E. *J. Biol. Inorg. Chem.* **2003**, *8*, 567.
39. Rodriguez-Lopez, J. N.; Tudela, J.; Varon, R.; Garcia-Carmona, F.; Garcia-Canovas, F. *J. Biol. Chem.* **1992**, *267*, 3801.
40. Dixon, M. *Biochem. J.* **1953**, *55*, 170.
41. Cabanes, J.; Chazarra, S.; García-Carmona, F. *J. Pharm. Pharmacol.* **1994**, *46*, 982.
42. Gąsowska, B.; Frąckowiak, B.; Wojtasek, H. *Biochim. Biophys. Acta* **2006**, *1760*, 1373.
43. Miiller, G. H.; Waldmann, H. *Tetrahedron Lett.* **1999**, *40*, 3549.
44. Horning, D. E.; Muchowski, J. M. *Can. J. Chem.* **1972**, *50*, 3079.
45. Sen, S.; Srivastava, B. B. L. *Indian J. Heterocycl. Chem.* **2005**, *14*, 269.
46. Ternce, C. O. J. *Heterocycl. Chem.* **1984**, *21*, 1573.
47. (a) Ainsworth, C. *J. Am. Chem. Soc.* **1958**, *80*, 5201; (b) Brown, D. J.; Cowden, W. B. *Aus. J. Chem.* **1983**, *36*, 1469; (c) Cheeseright, T. J.; Holm, M.; Lehmann, F.; Luik, S.; Marcia Gottert, M.; Melville, J. M.; Laufer, S. *J. Med. Chem.* **2009**, *52*, 4200; (d) Chande, M. S.; Barve, P. A.; Khanwelkar, R. R.; Athalye, S. S.; Venkataraman, D. S. *Can. J. Chem.* **2007**, *85*, 21; (e) Li, Y.; Liu, J.; Zhang, H.; Yang, X.; Liu, Z. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2278; (f) Joshi, S.; Karnik, A. V. *Synth. Commun.* **2002**, *32*, 111; (g) Maslat, A. O.; Abussaud, M.; Tashtoush, H.; Al-Talib, M. *Polish J. Pharmacol.* **2002**, *54*, 55; (h) Ainsworth, C. *J. Am. Chem. Soc.* **1955**, *78*, 4475; (i) Jansen, M.; Rabe, H.; Strehless, A.; Dieler, S.; Debus, F.; Dannhardt, G.; Akabas, M. H.; Lueddens, H. *J. Med. Chem.* **2008**, *51*, 4430; (j) Khan, K. M.; Rasheed, M.; Ullah, Z.; Hayat, S.; Kaukab, F.; Choudhary, M. I.; Atta-ur-Rahman; Perveen, S. *Bioorg. Med. Chem.* **2003**, *11*, 1381.
48. Hearing, V. J. *Methods Enzymol.* **1987**, *142*, 155.
49. Kahn, V.; Andrawis, A. *Phytochemistry* **1985**, *24*, 905.