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Thiosemicarbazide binds with the dicopper center in the competitive inhibition of mushroom tyrosinase enzyme: Synthesis and molecular modeling of theophylline analogues

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ABSTRACT

Theophylline is long known for its anti-ageing and anti-oxidative properties. Moreover, Tyrosinase is a crucial enzyme that regulates the melanin synthetic pathway, which is involved in various physiological metabolic processes including aging. The current paper describes the synthesis of various heterocyclic systems coupled with theophylline moiety along with their tyrosinase inhibition activity in view to identify the potent nucleus. Around 19 compounds were synthesized and screened for enzyme inhibition. Based on the current study, it is suggested that compound 18 having thiosemicarbazide has strong enzyme inhibition potential. The enzyme kinetics and docking studies provide important insights into how the compound interacts with the mushroom tyrosinase active site. The work will provide clue to developing new, potent tyrosinase inhibitors for drug development.

Tyrosinase is a crucial enzyme that regulates the melanin synthetic pathway, which is involved in various physiological metabolic processes and diseases, including aging, malignant cancer and neurodegeneration. The enzyme catalyzes the conversion of monophenols and *o*-diphenols to *o*-quinones in the Raper-Mason pathway of melanogenesis.[1–4] Hence, tyrosinase inhibitors possess skin-whitening and enzymatic browning inhibiting potentials, and thus draw the attention of medicinal chemists.[5] Inhibition of tyrosinase has clinical significance in many dermatological diseases or conditions such as post-inflammatory hyperpigmentation, melasma, flecks and nevus. A number of topical dermatological preparations use tyrosinase inhibitors such as kojic acid for skin-lightening and enhanced cosmetic effects.[6,7] Moreover, tyrosinase inhibition is also important for controlling undesired browning of fruits to increase their shelf life for commercial benefits.[8,9] But the lack of understanding of the interaction between enzyme and inhibitors limits the practical use of natural tyrosinase inhibitors in the cosmetics and pharmaceutical industries and further affects the development of new tyrosinase inhibitors.

Despite the limited progress made on tyrosinase inhibitors, the development methods which have both pharmacological profile and therapeutic safety represent a major concern for many scientists. Due to the wide range of properties associated with them, heterocyclic chemistry has generated intensive interest during the last many years. Several reports on the synthesis of heterocyclic molecules with enzyme inhibition potential have been published.[10] Moreover, theophylline is a natural product and is present in small quantities in tea leaves. It is an adenosine receptor blocker and commonly used in the prevention and treatment of asthma as bronchodilator. Recent reports suggest the use of theophylline having anti-ageing and anti-oxidative properties prompt us to explore it further. The current paper describes the synthesis of various heterocyclic systems coupled with theophylline moiety along with their tyrosinase inhibition activity in view to identify the potent nucleus. Therefore, there is a need to identify the core moiety responsible for such effects.

Purine heterocyclic system is considered as the most important natural ring system and being the parent ring in various analogues of biological importance.[11] Chlorotheophylline is a methylxanthine drug and closely resembles with caffeine. It is long considered for the treatment of vertigo, motion sickness and other pregnancy related disorders. It also suppresses the stimulation of certain nerves in the brain, thereby blocking the histamine receptors. The theophylline has varied physiological and pharmacological mode of action.[12] The most important

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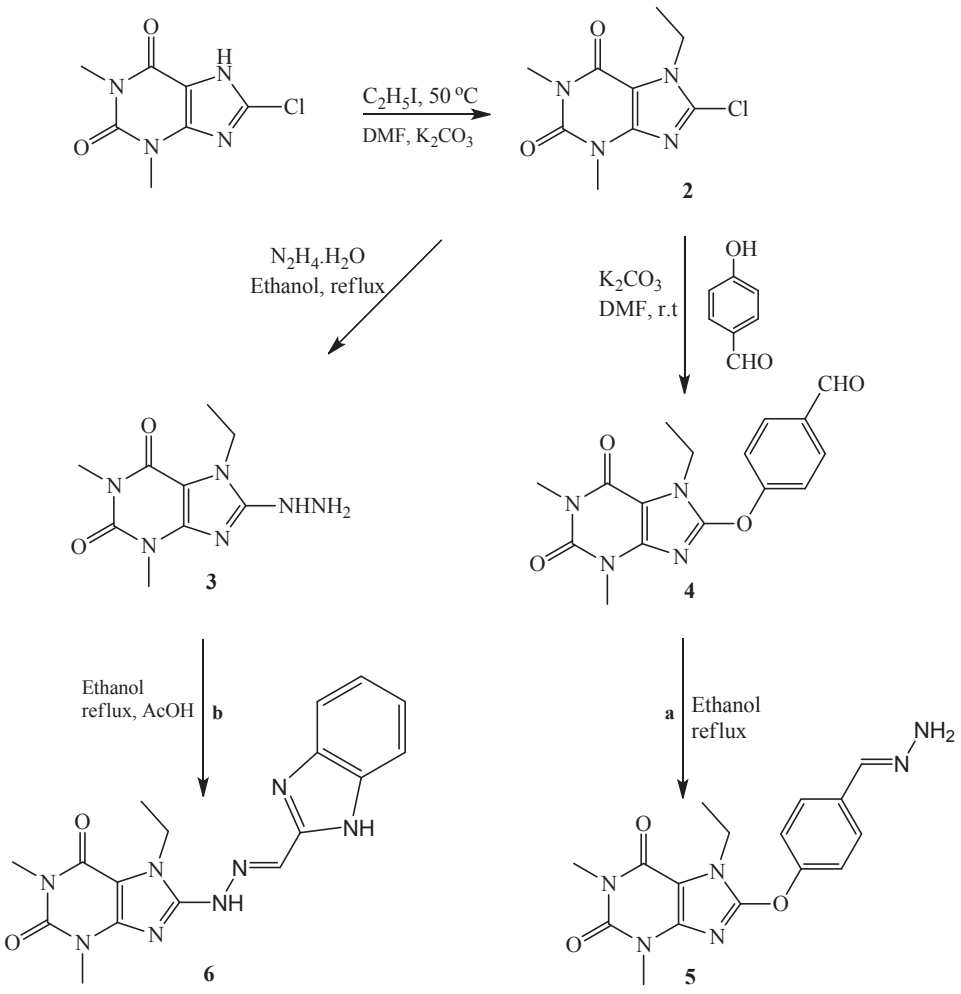


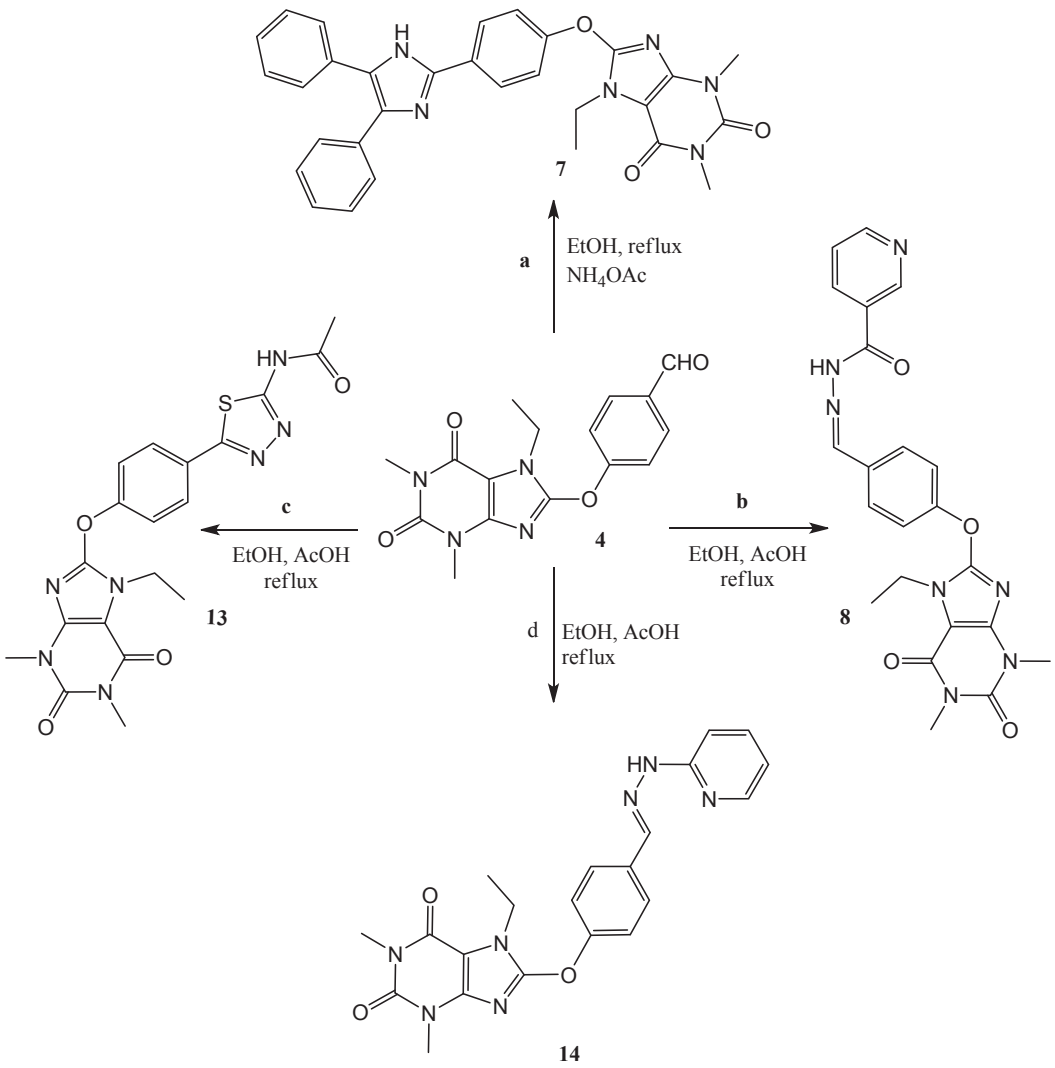
Scheme 1. Synthesis of starting materials a = hydrazinchydrate, b = 2-chloromethylbenzimidazole.

function of xanthines is to manage asthma exacerbations because of their ability to act directly on β-adrenergic receptors and relax bronchial smooth muscles. Owing to the considerable biological activities[13] associated with methylxanthine and in continuation to our interest in its chemistry, the present work describes the preparation of new theophylline analogues derived from the reaction of 8-chlorotheophylline with 4-hydroxyaldehyde and further.

Due to the presence of chloro group which can be replaced and leads to the formation of various precursors, is used as a starting material. 8-chlorotheophylline was treated in basic condition with ethyliodide in DMF as solvent afforded 8-chloro-7-ethyl-1,3-dimethyl-1*H*-purine-2,6 (3*H*,7*H*)-dione **2** in moderate yield (Scheme 1). This *N*-protection is necessary due to its acidic nature which prevents further modification on xanthine nucleus. The compound **2** was crystallized in ethanol and the crystallographic data was reported earlier by our group.[14]

The hydrazino derivative **3** of 7-ethyltheophylline was synthesized by refluxing with excess of hydrazine hydrate. Appearance of NH signals in the downfield region of ^{1}H NMR confirms the presence of desired molecule. The 7-ethyl-8-hydrazinothephyllin **3** was reacted with 2-chloromethylbenzimidazole in refluxing ethanol to get imidazole analogue **6** in good yield. The proposed molecule was confirmed by the presence of exocyclic CH proton around 8.25 ppm and the aromatic protons of benzimidazole moeity at around 7.2 ppm. For the synthesis of compounds **4**, 8-chloro-7-ethyl-1,3-dimethyl-1*H*-purine-dione were treated with 4-hydroxybenzaldehyde in the presence of anhydrous potassium carbonate in DMF (Scheme 1). The obtained theophylline carboxaldehyde **4** was reacted with various substituted hydrazines and isoniazid afforded schiff bases **5**, **8**, **14** and **16** in acidic medium (Scheme 2, 4). Disappearance of formyl and the presence of CH proton in the downfield region confirm the proposed structures. The mass and elemental analyses are also in accordance with the molecules.

Reaction with hydrazine-isatin derivative is of utmost importance because indoline-2-one molecules are well known for their antimicrobial properties. Disappearance of formyl proton and a presence of a singlet at ≈ 8 ppm for CH and a broad peak at 10.9 ppm in the ^{1}H NMR revealed the presence of NH and further confirm the postulated structure **15** (Scheme 4). Moreover, IR and mass spectra also confirms the proposed structures. Heterocycles containing imidazole ring system are found to exhibit a wide spectrum of biological activities including antibacterial and antiviral. Imidazole nucleus appears in a number of natural products like histidine and purine.[15] In an attempt to create imidazole ring system coupled with theophylline, **4** were reacted with benzil in the presence of ammonium acetate in refluxing acetic acid to produce **7** in quantitative yield (Scheme 2).



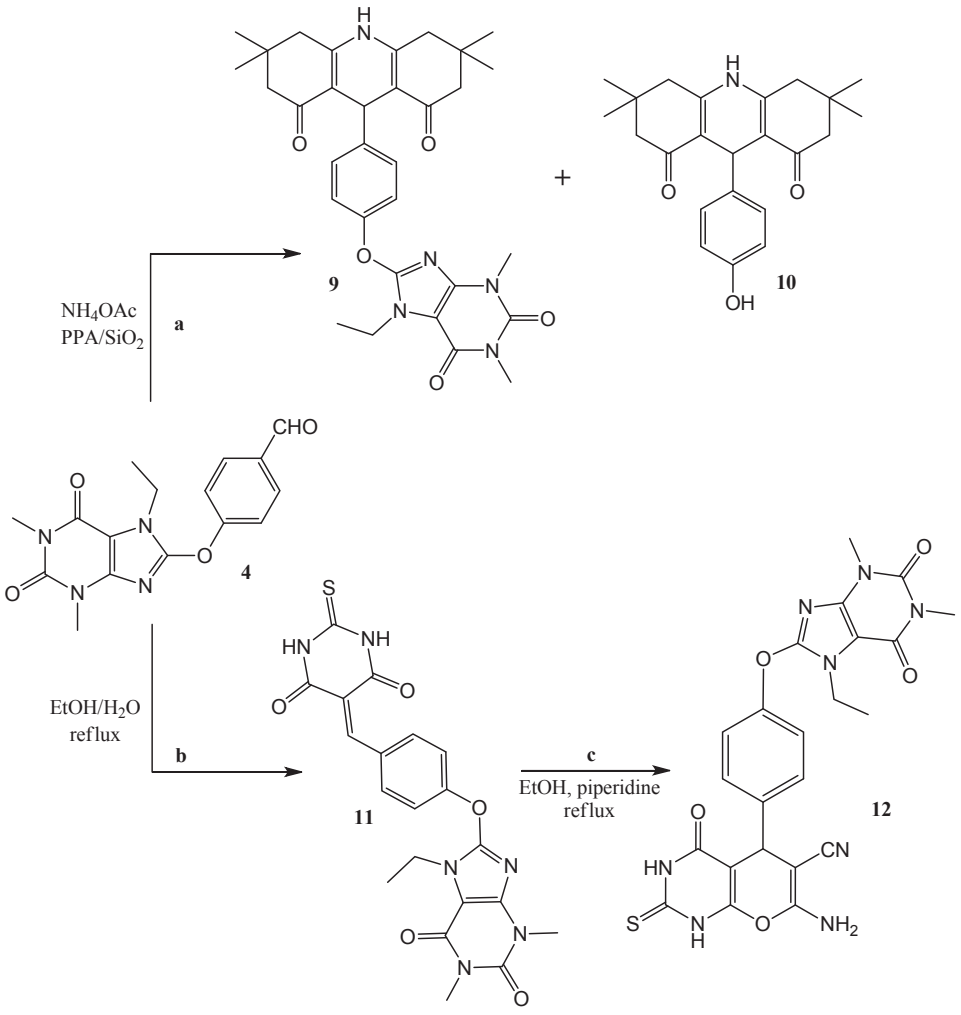
Scheme 2. Synthesis of 7-O alkylated products of theophylline a = Benzil, b = Nicotinichydrazide, c = Aceticanhydride, d = 2-hydrazinopyridine.

On refluxing a mixture of aldehyde **4** with dimedone and ammonium acetate in ethanol under conventional Hantzsch reaction conditions for a prolonged period of time was not successful due to the presence of various side products making it impossible to isolate the desired molecule.[16]

Therefore, for the synthesis of hexahydro acridine heterocyclic system coupled with theophylline base **9,** silica gel-supported polyphosphoric acid (PPA-SiO2) was used as a green and reusable heterogeneous catalyst.[17] In the course of the synthesis of **9**, a by-product **10** was obtained as a deprotected product and is assumed to be due to the Knoevenagel condensation which is an initial step in the cyclocondensation reaction. The NMR spectroscopic data are also consistent with the proposed structures; for example, compounds **9** and **10** exhibit in a proton NMR spectrum methyl singlet along with the doublet's peaks appear around 2-3 ppm corresponding to the diastereotopic CH_2 groups, respectively. In the current paper, construction of thiadiazole heterocyclic system was carried out by reaction of aldehyde **4** with thiosemicarbazide followed by reflux with aceticanhydride in acetic acid affording **13** in good yield (Scheme 2). More constrained thiazolidine ring system was selected due to their involvement in molecules reported as enzyme inhibitors. Reaction of thiobarbituricacid with aldehyde was carried out in green conditions using 20% water in ethanol afforded thiopyrimidine analogue **12** in quantitative yield. Cyclization was further achieved by reaction of **12** with malononitrile in catalytic amount of piperidine. The presence of CN in the ^{13}C NMR spectra at 116 ppm confirms the proposed molecule. Mass and elemental analysis further confirms the structure.

Push-pull butadienes are the versatile intermediates for the synthesis of various heterocyclic and natural products due to their biological prevalence and strong *pi-electron* interaction between donor and acceptor groups.[18] For this type of reactions Knoevenagel-Cope condition are the best choice but due to the tedious workup and lower yields we decided to use standard conditions.[19] Aldehyde **4** was stirred in ethanol at 50 °C overnight in the presence of anhydrous sodiumacetate yields the desired product **17** in moderate yield (Scheme 4).

Disappearance of formyl proton and the appearance of exocyclic

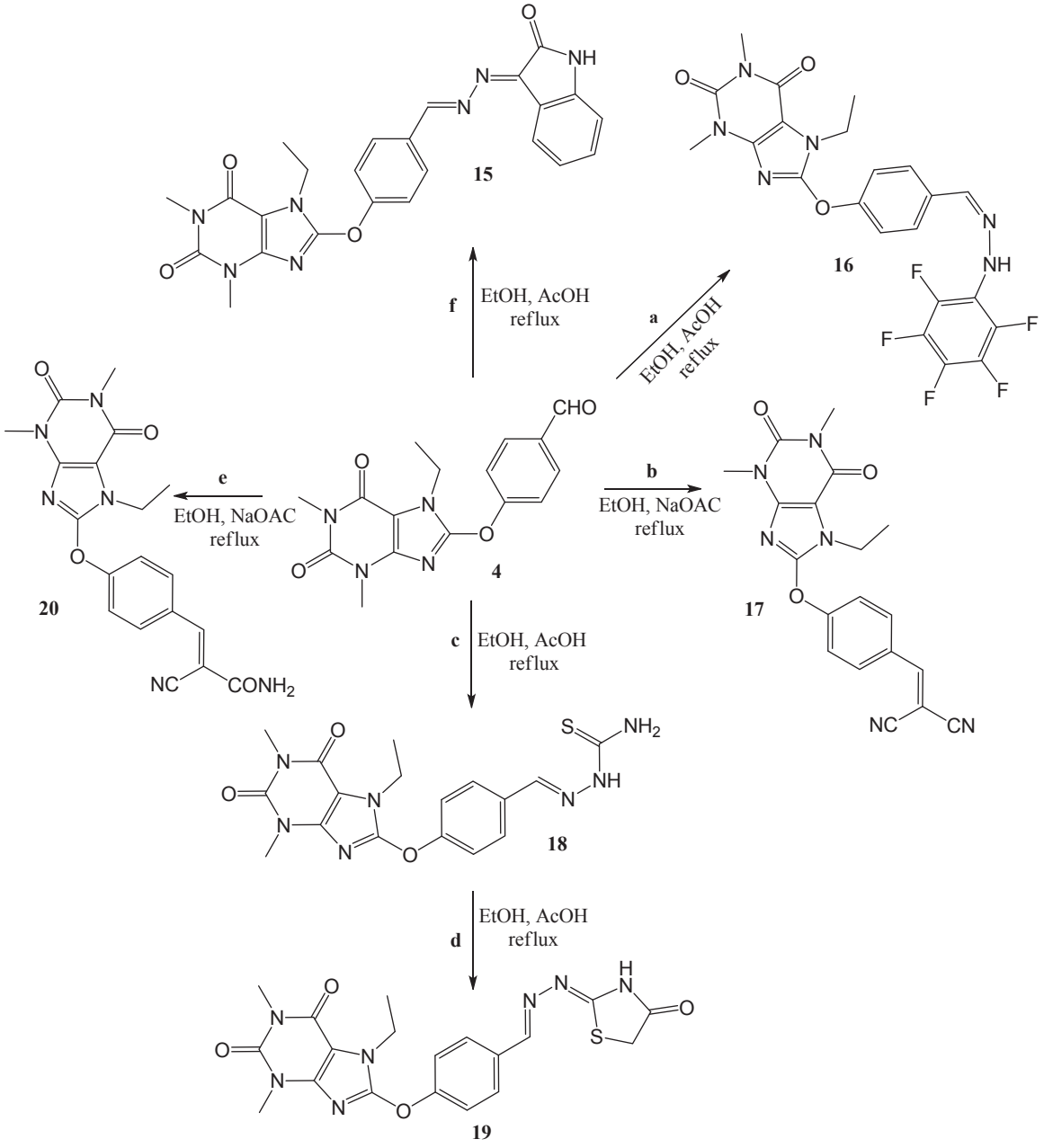


Scheme 3. Synthesis of 7-O alkylated analogues of theophylline a = Dimedone, b = Thiobarbituricacid, c = Malononitrile.

proton which corresponds to CH in the downfield region (≈8.2 ppm) on proton NMR confirm the proposed molecules. In order to obtain semicarbazide analogue, **4** was reacted with thiosemicarbazide in refluxing ethanol giving **18** in high yield. Construction of oxothiazolidine ring system **19** was carried out (Scheme 3) by reaction of **18** with chloroaceticacid in ethanol under reflux. Appearance of broad peak around 11.4 ppm corresponds to NH of thiazolidine ring confirms the proposed structure. All the structures were confirmed by one- and two-dimensional NMR spectroscopy.

All compounds decreased the rate of enzyme reaction compared to controls containing no inhibitors. Compound **18** was identified as most potent inhibitor of mushroom tyrosinase (K_i = 0.67 μM) that exhibited competitive inhibition of the enzyme. The Lineweaver-Burk plot for the compound is presented in Fig. 1. Other active compounds in the series (**4, 6, 8, 11, 12, 13, 19**) inhibited the enzyme in a range of 27.0–35% at 50 μM (Table 1). Although these compounds showed much weaker inhibitory activity than that of **18**, they provided important clues to redesigning new potent tyrosinase inhibitors. A general correlation of the size of the compounds with the inhibitory activity was observed. Compounds only with long side chains were found to be active; all other members in the series with short side chains were inactive. This is apparently explained by the crystal structure of mushroom tyrosinase that constitutes an active site with a large and deep binding cavity in which the dicopper center is located at its bottom.[20] A structurally longer molecule is necessary for approaching the dicopper center for inhibition. Interaction of inhibitors with the dicopper center is one factor that apparently contributes to potent inhibition of the enzyme in addition to binding to the cavity through other residues. Compound **18** bears all those structural features that are required for potent inhibition of the enzyme. It carries a longer side chain that can better reach down the deep active site pocket for binding. Additionally, it contains a thiourea group at the end of the side chain that is proposed to interact with the dicopper center. Our binding proposal for **18** was further confirmed by molecular docking studies (Fig. 2). The inhibitory activity of other compounds in the series indicates some sort of interaction with the enzyme albeit weak. Moreover, it is imperative that the enzyme is primarily selective to those compounds featuring long side chains. For the weak inhibitors identified in this work, further structural modifications such as structural extension, addition of thiourea or related groups would be necessary to ensure potent tyrosinase inhibition.

AutoDock Vina predicted a number of poses as indicated by a cluster of ligand conformations in the active site. Out of these, only those poses



Scheme 4. Synthesis of theophylline analogues a = Pentafluorophenylhydrazine, b = Malononitrile, c = Thiosemicarbazide d = Chloroaceticcid e = Cyanoacetamide, f = Isatinhydrazide.

were analyzed that were found to be based on previous knowledge of the crystal structures of PTU[21] and tropolone[22] in complex with potato catechol oxidase and mushroom tyrosinase, respectively. Each pose was analyzed for its score and presence of hydrogen bond interactions between the ligand and the enzyme. The pose with a score of – 6.30 was identified to be most favorable for such binding. The ligand strongly coordinated with the dicopper center of the active site in addition to hydrophobic interactions with various residues of the active site. However, none of the poses revealed hydrogen bond interactions with the active site residues.

The active site of the enzyme is located in the surface of the *H* subunit and is accessible from the solvent. It is surrounded by four antiparallel α-helices forming a large, deep pocket with the dicopper center at its bottom featuring a planar trigonal geometry. Each of the copper atoms is coordinated by three histidine residues; CuA by His 61, 85, 94 and CuB by His 259, 263, 296. In the native crystal structure of mushroom tyrosinase, a water molecule forms a bridge between the two copper atoms yielding a trigonal pyramidal coordination sphere. Compound **18** binds to the active site in a productive manner and fits well into the long, deep pocket with its thiourea group interacting with the dicopper center.

The overall molecular length of the compound is decisive for such binding, enabling access to the important interaction with the dicopper center. The sulfur atom of the thiourea group strongly coordinates with both CuA and CuB of the center forming a trigonal pyramidal coordination sphere. This type of coordination is either a strong electrostatic interaction or even a covalent bond (Cu–S bond distance 2.20 Å). The

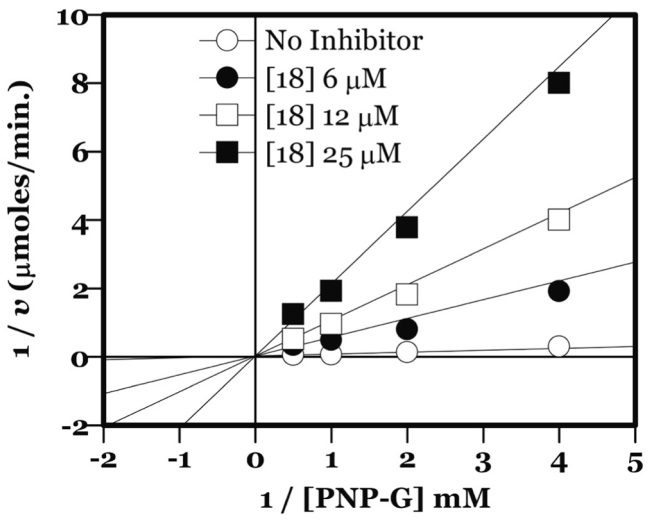


Fig. 1. Lineweaver Burk plot for compound **18** exhibiting competitive inhibition of mushroom tyrosinase.

Table 1
Mushroom tyrosinase inhibitory activities of the target compounds.

Compound	% Tyrosinase inhibition at 50 μM
4	29.51 ± 1.18
6	27.01 ± 1.40
8	40.30 ± 2.81
11	30.25 ± 1.36
12	32.68 ± 1.60
13	33.61 ± 1.82
18	**K_i = 0.67 ± 0.03 μM**
19	29.40 ± 1.25
3, 5, 7, 9, 14, 15, 16, 17, 18	>40
Kojic acid	98.75 ± 2.54

thiourea moiety has rotated in order to approach the dicopper center (Fig. 2).

The binding is very similar to that of PTU binding to potato catechol oxidase as revealed in its crystal structure since its active site shares significant homology to that of mushroom tyrosinase (Fig. 3). However, the binding of compound **18** is unlike that of tropolone which interacts with the active site in an unproductive manner (Fig. 3). The compound forms no hydrogen bonds with the active site residue; however, it is involved in multiple hydrophobic interactions with Phe 264, Arg 268 and Val 283 residues (Table 2). The benzene ring of the compound is in proximity to Val 283 making hydrophobic interactions with it. Moreover, the xanthine moiety of the compound is oriented in a position that appears to be protruding out of the enzyme surface and at the entrance of the active site pocket. It is mainly involved in hydrophobic interactions with Phe 264 and Arg 268 residues (Fig. 2). The enzyme kinetics and docking studies provide important insights into how these compounds interact with the mushroom tyrosinase active site. The work will provide clue to developing new, potent tyrosinase inhibitors for drug development.

Conclusion

Based on the molecular docking and enzyme inhibition data it is observed that the sulfur atom of the thiourea group is vital and it strongly coordinates with both CuA and CuB of the enzyme center forming a trigonal pyramidal coordination sphere. The thiourea moiety has rotated in order to approach the dicopper center making the molecule a strong drug candidate. Accordingly, it could be concluded that the presence of H atom at position *N*-7 of purine is essential for further reactions and biological activity due to its acidic nature.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

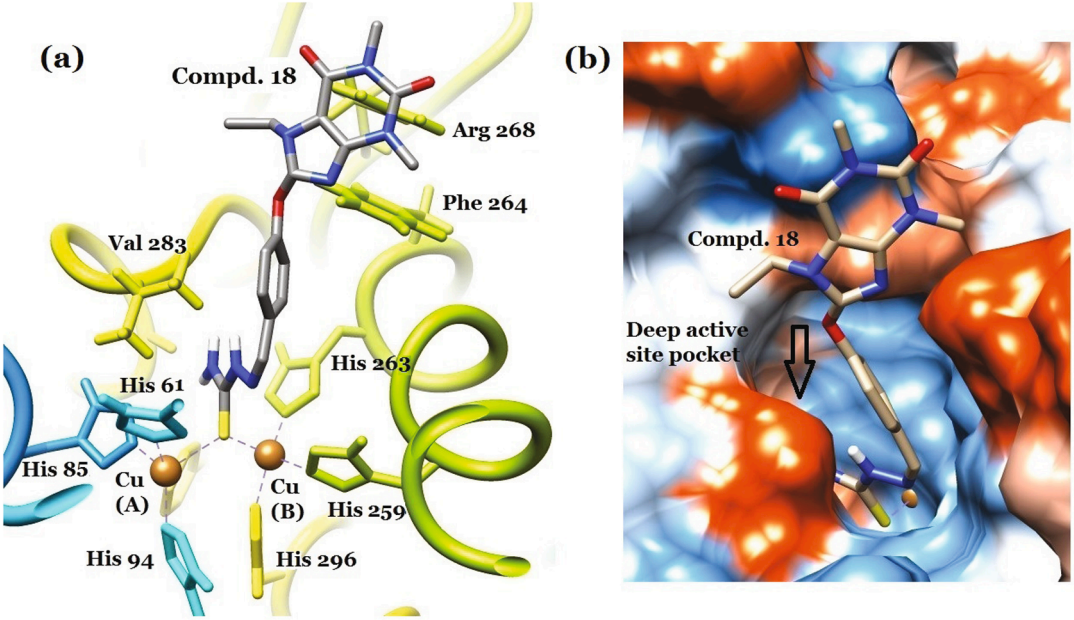


Fig. 2. (a) Compound **18** docked into the mushroom tyrosinase active site. The thiourea group coordinates with the dicopper center forming a trigonal pyramidal coordination sphere. Its benzene and xanthine moieties are stabilized by hydrophobic interactions with Phe 264, Arg 268 and Val 283 residues. (b) Surface diagram of the long, deep active site pocket of the enzyme where the inhibitor binds. Hydrophobic residues are shown in orange. [Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311].

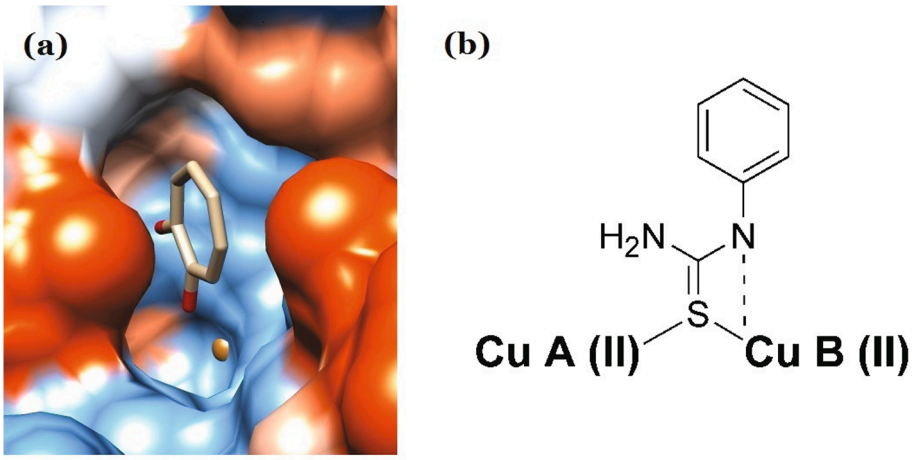


Fig. 3. (a) Crystal structures of (a) tropolone in complex with mushroom tyrosinase and (b) PTU in complex with potato catechol oxidase for comparative purposes. The binding of compound 18 to the dicopper center is very similar to that of PTU complex. [Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311].

Table 2
Hydrophobic interactions between compound **18** and the enzyme obtained from PLIP server.

Residue #	Amino acid	Distance (Å)
264A	Phe	3.59
283A	Val	3.66
268A	Arg	3.20

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Appendix A. Supplementary data

The copies of ^{1}H NMR, ^{13}C NMR and Mass spectra for all new synthesized compounds have been submitted along with the manuscript. Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.127826.

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