Synthesis and structure-activity relationship of tyrosinase inhibiting novel bi-heterocyclic acetamides: Mechanistic insights through enzyme inhibition, kinetics and computational studies

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ABSTRACT

The present research was designed for the selective synthesis of novel bi-heterocyclic acetamides, \(9a-n\), and their tyrosinase inhibition to overwhelm the problem of melanogenesis. The structures of newly synthesized compounds were confirmed by spectral techniques such as \(^1\)H NMR, \(^{13}\)C NMR, and EI-MS along with elemental analysis. The inhibitory effects of these bi-heterocyclic acetamides (\(9a-n\)) were evaluated against tyrosinase and all these molecules were recognized as potent inhibitors relative to the standard used. The Kinetics mechanism was analyzed by Lineweaver-Burk plots which explored that compound, \(9h\), inhibited tyrosinase competitively by forming an enzyme-inhibitor complex. The inhibition constants \(K_i\) calculated from Dixon plots for this compound was 0.0027\(\mu\)M. The computational study was coherent with the experimental records and these ligands exhibited good binding energy values (kcal/mol). The hemolytic analysis revealed their mild cytotoxicity towards red blood cell membranes and hence, these molecules can be pondered as nontoxic medicinal scaffolds for skin pigmentation and related disorders.

1. Introduction

Heterocyclic compounds are prevalent in various fields of life sciences. These compounds carry out several significant tasks in technology, nature and medicine. Heterocyclic hybrids have become most promising molecules due to its numerous potential as novel drug candidates in modern drug discovery. It is a challenging and critical issue for the pharmaceutical industry and academic researchers to develop new drugs and pharmaceuticals [1,2]. Heterocyclic compounds have wide range of medicinal properties which inspired Chemists to synthesize their new hybrids and screen its pharmacophoric properties. Heterocyclic compounds containing nitrogen, especially azoles, and sulfur have attained constant interest in organic synthesis because they are pharmacologically active and important pharmaceutically [3–5].

Thiazoles are familiar aromatic five member ring heterocyclic compound containing both sulfur and nitrogen atom in the ring and their utility as medicaments is very much established. They are fundamental part of all the available penicillins which have revolutionized the therapy of bacterial diseases [6]. A literature survey revealed that derivatives of thiazoles have numerous biological activities such as antimicrobial [7,8], anticancer [9–12], antifungal [13], anti-inflammatory [14], anti-hypertension [15], antitubercular [16], antioxidant [17], analgesic [18], and anthelmintic [19] activities.

Abbreviations: IR, Infra Red; EI-MS, Electron Impact Mass Spectrometry; \(^1\)H-NMR, Proton Nuclear Magnetic Resonance; \(^{13}\)C-NMR, Carbon Nuclear Magnetic Resonance; DMSO-\textsubscript{d\textsubscript{6}}, Deuterated dimethyl sulfoxide; \(s\), singlet; \(d\), doublet; \(dd\), doublet of doublets; \(t\), triplet; \(br\), broad triplet; \(q\), quartet; \(quint\), quintet; \(sex\), sextet; \(sep\), septet; \(m\), multiplet

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Triazoles are five membered heterocyclic compounds containing two carbons and three nitrogen atoms. These molecules have maintained a unique position in medicinal and organic chemistry due to their numerous biological activities. The literature survey shows that derivatives of triazole contain extensive range of pharmacological activities such as antioxidant [20], antimicrobial [21,22], antiviral [23], anti-inflammatory, local anaesthetic [24], antimalarial [25], analgesic [26], antineoplastic [27], antiproliferative [28], and anticonvulsant [29]. Many of the triazole based derivatives are now available as medicines [30].

Tyrosinase, also known as polyphenol oxidase enzyme, is widely distributed in nature including higher plants, fungi, bacteria and animals [31]. It is responsible for browning of vegetables, beverages, fruits and various other products. It catalyzes the hydroxylation of monophenols to ortho-diphenols and subsequently oxidizes the products to corresponding ortho-quinones by using molecular oxygen [32]. Champignon mushroom, Agaricus bisporus is a source of tyrosinase. Tyrosinase is extracted from it and is well suited as a model for the studies on melanogenesis. Mushroom tyrosinase is the only commercially available enzyme and therefore almost all the tyrosinase inhibitory studies are conducted on it [33].

Human skin is frequently exposed to ultraviolet radiation, which is an induction factor of reactive oxygen species. Formation of pathological skin pigmentation or direct DNA damage is the cause of excessive reactive oxygen species and leads to induce skin injury. Antioxidant defense system helps oxidative stress [34–36]. Prevention of damage due to ultraviolet radiation to skin pigmentation is also done by another mechanism. These ultraviolet radiations are being absorbed by Melanin thus protecting skin cells from them [37]. Therefore, for human health, normal skin pigmentation is very essential [38]. Tyrosinase is a copper containing enzyme and is essential in the melanin biosynthesis and therefore, responsible for pigmentation of skin and hair in mammals [39–41]. Apart from the production of melanin, tyrosinase has many other functions including detoxification of host plant defensive phenols for symbiotic bacteria [42–43] and synthesis of amino acid based antibiotics [44].

Although some azole derivatives have been recently reported as tyrosinase inhibitors [45–48], yet there is a need to discover some unique and safe inhibitors of this enzyme to devastate the problems of melanogenesis. Therefore, in continuation of our previous studies on bioactivity of related bi-heterocyclic bi-amides [49], the present investigation was designed to seek some novel 2-aminothiazole-phenyl-triazole hybrid molecules bearing N-(aralkyl/aryl)acetamides as tyrosinase inhibitors.

2. Results and discussions

2.1. Chemistry

The targeted bi-heterocyclic N-substituted acetamides were synthesized in several steps as delineated in schemes 1a and 1b and the varying groups have been listed in Table 1. The synthesis was planned by refluxing ethyl 2-(2-amino-1,3-thiazol-4-yl)acetate (1) in methanol and hydrazine hydrate to convert it to 2-(2-amino-1,3-thiazol-4-yl)aceto hydrazide (2). The hydrazide, 2, was taken in methanol and refluxed with phenyl isothiocyanate (3) to transform it into a solid intermed iary compound 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-N-phenyl-1-hydrazinecarbothioamide (4) which was cyclized further to yield the target triazole hybrid molecule and hydrazine hydrate to convert it into 2-(2-amino-1,3-thiazol-4-yl)acetamides (8a-n).

To attain the peak success in this synthesis, the nucleophile 5 was dissolved in DMF and a pinch of LiH was added into it. The mixture was stirred for about 30 min for activating its mercapto position, and then in this final step, various electrophiles, 8a–n, were coupled in equimolar amounts with the activated 5 to yield the targeted bi-heterocyclic molecules, 2-{(5-[2-(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl)sulfonyl}-N-(aralkyl/aryl)acetamides (9a–n).

The morphology of the synthesized compounds was well justified as well as their structures were corroborated with aid of spectral data obtained by the detailed spectroscopic studies. Structural analysis of one compound is discussed hereby for the benefit of readers. The molecule 9f was synthesized as bright yellow amorphous solid having melting point of 100–101°C. Its molecular formula, C22H22N6OS2, was recognized by CHN analysis data and molecular mass was justified by the molecular ion peak in its EI-MS at m/z 450. The mass fragmentation pattern also supported to erect the said molecular formula. Moreover, counting the number of protons in its 1H NMR spectrum and the carbon resonances in its 13C NMR spectrum, also augmented this assignment.

IR spectrum was used to recognize various functionalities in the molecule. The characteristic peaks appeared at v 3358 (N–H stretching), 3048 (C–H stretch of aromatic ring), 2922 (–CH2– str.), 1642 (C=O stretching), 1578 (C=C stretch of aromatic ring), 1521 (C=N stretch), 1167 (C–N–C bond stretch), and 625 cm–1 (C–S stretch). In its 1H NMR spectrum, the phenyl ring linked with nitrogen (4') of 1,2,4-triazole heterocyclic ring was identified by two prominent multiplets in the aromatic region at δ 7.52–7.50 (m, 3H, H-3', H-4' & H-5') and 7.37–7.35 (m, 2H, H-2' & H-6'). A 4-phenylthio moiety attached to nitrogen of acetylated unit was clearly identified by A2B2 spin system in aromatic region, represented by two ortho-coupled broad doublets at δ 7.45 (br.d, J = 8.5 Hz, 2H, H-2'' & H-6''), and δ 7.14 (br.d, J = 7.14 Hz, 2H, H-3'' & H-5'') along with characteristic quartet and a triplet for ethyl group at δ 2.55 (q, J = 7.6, 2H, CH3-CH2-4''), and 1.15 (t, J = 7.6, 3H, CH3-CH2-4''). The 2-amino-1,3-thiazol-4-yl moiety was characterized by two singlets at δ 6.84 (br.s, 2H, H2-N2), and δ 5.85 (s, 1H, H-5), while a singlet δ 3.77 (s, 2H, CH2-N) was assignable to a methylene group linking the two heterocycles in the molecule. The acetylated unit in the molecule was unified by two peculiar singlets at δ 10.24 (s, 1H, –CO-NH-CO–), and δ 4.10 (s, 2H, CH2-2''). The 1H NMR spectrum of this molecule has been shown in Fig. S1(a). Its expanded aromatic region has been shown in Fig. S1(b) and its expanded aliphatic region has been presented in Fig. S1(c).

The 13C NMR spectrum (Fig. S2) was also used to substantiate all the assignments. Overall, eighteen carbon resonances were observed due to some symmetrical carbons in the molecule. The 2-amino-1,3-thiazol-4-yl moiety was signified by two quaternary signals at δ 168.20 (C-2), and 145.60 (C-4), besides a methane signal at δ 102.18 (C-5). Likewise, the other heterocyclic, i.e. 1,2,4-triazole-5-yl)sulfonyl was also rationalized by two quaternary signals at δ 153.39 (C-5'') and 149.71 (C-3') while a methylene joining the two heterocycles (4-position of the former heterocycle with 5''-position of the latter heterocycle) was obvious at δ 27.48 (C-6). The 4-ethylphenyl moiety was apparent with two quaternary signals at δ 138.89 (C-1''), and 136.43 (C-4'') along with two symmetrical methine duplets at δ 127.92 (C-3'' & C-5'') and δ 119.12 (C-2'' & C-6''). The 4-ethyl group was easily characterized by δ 27.55 (CH3-CH2-4''), and δ 15.61 (CH3-CH2-4''), and the central acetylated unit was corroborated by a downfield carbonyl signal at δ 165.27 (C-1'') and a methane signal at δ 35.93 (C-2''). The phenyl ring attached to the nitrogen (4') atom of 1,2,4-triazole ring was recognized by a quaternary signal at δ 132.90 (C-1''), and three methane resonances at δ 129.78 (C-4''), 129.55 (C-3'' & C-5'') and 127.14 (C-2'' & C-6''). Various connectivities in the carbon skeleton were thoroughly ascertained by its HMBC spectrum. This spectrum, along with important correlations, is shown in Fig. S3. So, on the basis of aforementioned accumulative evidences, the structure of 9f was confirmed and it was named as 2-{(5-[2-(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl)sulfonyl}-N-(4-ethylphenyl)acetamide. A similar protocol was exercised for the structural characterization of other synthesized molecules (Figs. S4–S17).
2.2. Tyrosinase inhibition and structure-activity relationship

The newly synthesized bi-heterocyclic acetamides (9a-n) were evaluated for their inhibitory potentials against tyrosinase enzyme and their results are tabulated in Table 2. All these compounds exhibited very potent inhibitory activities against this enzyme, as evident from their lower IC$_{50}$ (µM) values, relative to standard, kojic acid, having IC$_{50}$ value of 16.8320 ± 1.1600µM. Though the rendered activity is accumulative of a whole molecule, yet a limited structure-activity relationship (SAR) was recognized by examining the effect of different aryl entity on the inhibitory potential, as it was the only varying part and all other parts were intact in all molecules. The general structural

![Scheme 1a](image1)

**Scheme 1a.** Outline for the synthesis of 2-{5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl}sulfanyl-N-(aralkyl/aryl)acetamides. **Reagents & Conditions:** (A) MeOH/N$_2$H$_4$·H$_2$O/refluxing for 2 hrs. (B) MeOH/3/Refluxing for 1 hr. (C) The ppt. of 4 dissolved in 10% NaOH/filtration/acidification of filtrate in cold state to get ppt. of 5. (D) DMF/LiH/stirring for 10–12 hrs.

![Scheme 1b](image2)

**Scheme 1b.** Synthesis of various electrophiles involved in Scheme 1a. **Reagents & Conditions:** (E) Aq. Na$_2$CO$_3$ soln./pH 9–10/vigorous manual shaking at RT for 2–3 hrs.

2.2. Tyrosinase inhibition and structure-activity relationship

The newly synthesized bi-heterocyclic acetamides (9a-n) were evaluated for their inhibitory potentials against tyrosinase enzyme and their results are tabulated in Table 2. All these compounds exhibited very potent inhibitory activities against this enzyme, as evident from their lower IC$_{50}$ (µM) values, relative to standard, kojic acid, having IC$_{50}$ value of 16.8320 ± 1.1600µM. Though the rendered activity is accumulative of a whole molecule, yet a limited structure-activity relationship (SAR) was recognized by examining the effect of different aryl entity on the inhibitory potential, as it was the only varying part and all other parts were intact in all molecules. The general structural
parts of the studied acetamides are featured in Fig. 1.

Among the following four di-methylated regio-isomers, compound 9a in which the two methyl groups were present on adjacent positions (2,3-dimethylphenyl), and probably due to steric crowding it exhibited least inhibitory activity (IC50 = 2.0181 ± 0.1629 µM) as compared to other isomers. Even it was even least active in the whole series, yet it was much better inhibitor than standard, kojic acid (IC50 = 16.8320 ± 1.1600 µM). The other analogues 9b, 9c and 9d possessed very resembling inhibitory potential with IC50 values of 0.0547 ± 0.0211, 0.0589 ± 0.0011 and 0.0513 ± 0.0035 µM respectively, indicating that separation of the methyl groups probably augmented the suitable interactions with the active site of the enzyme (Fig. 2).

Contrary, when the inhibitory potential of other two isomers was compared it was observed that 9e with adjacent methyl groups (3,4-dimethylphenyl), had a better activity (IC50 = 0.1133 ± 0.0038 µM), relative to 9f (IC50 = 0.2465 ± 0.0019 µM), probably due to the reason that the hereby adjacent methyl groups in 9e were away from the point of attachment of aromatic ring, thus the same steric factor was diminished to some extent and this molecule became comparatively more suitable for inhibition of tyrosinase enzyme (Fig. 3).

When the inhibitory potential of ortho-substituted molecules was compared, it was revealed that 9g with a smaller methyl group was more prone to the inhibition of tyrosinase (IC50 = 0.0156 ± 0.0021 µM). The presence of little bulky ethyl group at ortho-position retarded the activity of 9h (IC50 = 0.1156 ± 0.0021 µM) and a further decrease was observed in 9i (IC50 = 0.1943 ± 0.0063 µM) in which both ortho-positions were occupied, one with a smaller methyl group and other with a little bulky ethyl group (Fig. 4). It means that the presence of only smaller group/s at ortho-position can favour the inhibitory activity in these molecules and indeed 9g was the most active compound in the synthetic series.

In between the para-substituted molecules, 9j with 4-ethyl group in aryl part exhibited slightly better inhibitory potential (IC50 = 0.1224 ± 0.0023 µM) as compared to 9k (IC50 = 0.1726 ± 0.0149 µM) in which 4-ethoxy group was present (Fig. 5). It means the presence of a slightly bulky polar group was not a suitable entity to impart a superb inhibitory activity to a molecule.

A very interesting trend was observed in the inhibitory activity of molecules with an un-substituted aryl part. The insertion of one methylene group in between nitrogen atom and phenyl ring in 9m (IC50 = 1.0108 ± 0.0614 µM) decreased its activity relative to 9l (IC50 = 0.1330 ± 0.0129 µM) in which no such methylene was present. However, the presence of two methylene in 9n (IC50 = 0.0161 ± 0.0014 µM) enhanced its activity, probably due to more flexibility in the aryl part of the molecule and it behaved as a promising inhibitor and was indeed the second most active compound in the series (Fig. 6).

So, it was envisaged from the structure-activity relationship that in the current synthetic series, the molecules, either bearing small sized
2.3. Kinetic analysis

To understand the inhibitory mechanism of synthetic bi-hetero-
cyclic acetamides on tyrosinase inhibition, kinetic study was per-
formed. Based on our IC50 results, we selected the most potent com-
pound 9g to determine their inhibition type and inhibition constant.
The kinetic results of the enzyme by the Lineweaver-Burk plot of 1/V
versus 1/[S] in the presence of different inhibitor concentrations gave a
series of straight lines, the result of Lineweaver-Burk plot of compound
9g showed that Vmax remains the same without significantly affecting
the slopes. Km increases with increasing concentration, while Vmax re-
mains the same with an insignificant difference. This behavior in-
dicated that 9g inhibited the enzyme tyrosinase in a competitive
manner (Fig. 7; (A)). The second plot (Fig. 7; (B)) of slope against the
concentration of 9g showed EI dissociation constant. Ki was calculated
from the inhibitor concentration of 9g versus the slope and Ki was
found to be 0.0027 µM.

2.5. Computational analysis

2.5.1. Bio-chemical properties and lipinski’s rule of five (RO5) validation

The biochemical properties of all the synthesized chemical com-
pounds, 9a-n, were predicted by using computational servers and tools
and validated through Lipinski Rule of five (RO5) analysis. The RO5
states as compounds must contain < 500 (g/mol) molecular mass
and < 5 logP values, respectively. Moreover, the compounds should
possess no > 10 hydrogen bond acceptors (HBA) and 5 hydrogen bond
donor (HBD), respectively. The molecular weight (g/mol), logP, HBA/D
all compounds, 9a-n, were not exceeded then standard value as men-
tioned in Table 3. The exceed values of HBA and HBD results in poor
permeation [50]. The hydrogen-bonding capacity has been considered
as significant parameter for drug permeability. Our predicted results
showed that all the synthesized compounds were present in standard
range and obeyed the RO5 rule. Polar surface area (PSA) is also con-
sidered as good descriptor for characterizing the drug absorption, in-
cluding intestinal absorption, bioavailability and blood-brain barrier

group/s at ortho-position/s in the aryl part like 9g and 9d or a molecule
with a flexible aryl part like 9n, generally behaved as highly potent
inhibitors of the tyrosinase enzyme.

2.4. Hemolytic activity

The synthesized compounds, 9a-n, were also exposed to hemolytic
assay to ascertain their safe utility as therapeutic agents. The cyto-
toxicity was profiled a percentage results of hemolysis and the results
are shown in Table 2. It was obvious from the results that all derivatives
of this series have modest toxicity towards red blood cell membrane.
Maximum membrane toxicity was shown by the compound 9i
(21.4 ± 0.05%) which is much lower than the positive control,
Triton X (89.11 ± 0.01%). The minimum toxicity was rendered by 9d
(4.11 ± 0.02%) in the series. In general, it was rational that most of
the molecules exhibited very moderate cytotoxicity and thus can be
considered for further applications in drug designing programs.

Fig. 2. Structure-activity relationship of compounds 9a, 9b, 9c, and 9d.

Fig. 3. Structure-activity relationship of 9e and 9f.
Our predicted results showed that all compounds, 9a-n, possess < 140 Å² PSA values which may enhance their absorption efficacy in the body. The computational drug-likeness prediction is a combination of various molecular properties such as hydrophobicity, electronic distribution, hydrogen bonding characteristics, molecule size with flexibility and presence of various pharmacophoric features [52]. Our results showed that synthetic compounds, 9a-n, showed good drug score values except 9e which showed negative results. The positive values of synthetic compounds showed good drug-likeness behavior whereas, compounds with negative values have less drug-likeness probability (Table 3).

2.5.2. Pharmacokinetic assessment of synthesized compounds

The pharmacokinetics properties such as absorption, distribution, metabolism, excretion and toxicity (ADMET) of newly synthesized compounds are considered as major hallmark to predict the lead structures efficacy. The absorption properties like water absorption (log mol/L), intestinal solubility (% absorbed) and skin permeability (logKp) values predict the therapeutic potential of newly synthesized compounds. The drug absorption is depends upon routes of administration either orally, intravenously or subcutaneously and chemical compounds target a specific tissue by using bloodstream pathway. Prior research data showed that ligands having good absorption value have more chance to cross gut barrier by passive penetration [53]. The water solubility results justified that compounds, 9a-n showed good absorption and may have potential to cross gut barriers. Furthermore, all compounds, 9a-n, exhibited good intestinal solubility results which are comparable to standard value (> 30 %abs). The skin permeability values of all compounds, 9a-n, were also present in the acceptable range compared with standard value (−2.5 logKp) which predict their drug likeness behavior. The compounds having < 30% absorption may not considered as good lead like structure. Moreover, Blood Brain Barrier (BBB) and Central Nervous System (CNS) permeability values of all compounds were also acceptable with the standard values (0.3 to < −1 log BB and > −2 to < −3 logPS), respectively. Results showed that compounds displayed a comparable predicted values with standard and have potential to cross the barriers and to target the receptor. Moreover, their metabolic behavior was confirmed by CYP1A2, CYP2C19 and CYP2C9 inhibitor, which are isof orm of cytochrome P450. Results showed the compounds possessed good inhibitory behavior for CYP2C19 and CYP2C9, however, for CYP1A2 compounds (9h-I, 9k and 9n) were beyond the inhibition behavior. The excretion and toxicity predicted values were also justified the drug likeness behavior of these compounds on the basis of total clearance (log ml/min/kg), AMES toxicity, maximum tolerated dose (MTD) and LD50 values. The non-mutagenic and non-toxic behaviors of synthesized compounds were observed from AMES toxicity prediction. Compounds showed hepatotoxic behavior while no one showed skin sensitization effects. The predicted ADMET properties justified that these novel synthesized compounds may have good lead like potential with mild hepatotoxic effects (Table 4).

2.5.3. Molecular docking and binding energy analyses

Molecular docking experiment is best approach to study the binding conformation of ligands within the active region of target proteins [54,55]. The docked complexes of synthesized compounds, 9a-n, against mushroom tyrosinase were analyzed on the basis of lowest binding energy values (kcal/mol) and hydrogen/hydrophobic

Fig. 4. Structure-activity relationship of 9g, 9h and 9i.

Fig. 5. Structure-activity relationship of 9j and 9k.
interaction pattern. Results showed that all the ligands, 9a-n, exhibited good docking energy values and showed their interaction within active region of target protein (Table 5). GlideScore is based on ChemScore (fitness function), but includes a steric-clash term, adds buried polar terms devised by Schrödinger to penalize electrostatic mismatches. The GScore is calculated as from the Eq. (1).

\[
GScore = vdW + Coul + Lipo + Hbond + Metal + BuryP + RotB + Site...
\]

(1)

\(vdW =\) Van der Waals energy, \(Coul =\) Coulomb energy, \(Lipo =\) Lipophilic, \(Hbond =\) Hydrogen-bonding, \(Metal =\) Metal-binding, \(BuryP =\) Penalty for buried polar groups, \(RotB =\) Penalty for freezing rotatable bonds and \(Site =\) Polar interactions in the active site. Based on in vitro and in silico docking energy results, 9g was ranked as best ligands which showed good inhibitory potential against targeted enzyme as compared all other derivatives. Although, the basic nucleus of all the synthesized compounds were same, therefore most of compounds possess good efficient energy values and have no big energy fluctuations difference.

2.5.4. Binding pocket and ligands binding conformations

The binding pocket analysis showed that ligands, 9a-n, were confined in the active region of target protein. All three docked structures were superimposed to check their binding configuration in the active region of target protein. Results showed that synthesized compounds were bound in the binding pocket having similar conformational pattern (Fig. 8).

2.5.5. Binding analysis

The docked complexes were analyzed on the basis of bonding interactions pattern. Based on in vitro and docking energy results, the most promising compound (9g) was selected to check its conformational position inside the active region of target protein (Fig. 9). The 2-methyphenyl ring of 9g formed a couple of hydrophobic interactions at...
His85 and His259 residues within the active region of target protein. Both residues are metal bound residues and play a significance in protein stability. Similarly, another phenyl ring is attached with His244 and 2-aminothiazole ring is with His85. The aminogroup of fivethiazol ring showed another significant interaction with Cys83. The already published computational data results showed good correlation with our docking results [56,57]. The 2-dimensional graphical depiction of docking complex is mentioned in Fig. 10, whereas all others are listed in supplementary data Figs. S18-S30.

3. Conclusion

A structurally distinct series of novel molecules, amalgamated with a thiazole, a triazole and an acetamide moiety, was synthesized and recognized with very superb tyrosinase inhibition. It was postulated from their SAR studies that molecules particularly bearing small sized methyl group/s at ortho-position/s in aryl part or a compound with a flexible phenethyl group, generally inhibited the tyrosinase in an excellent manner. All these molecules also showed mild cytotoxicity towards red blood cell membranes. Therefore, it was pertinent to conclude that these bi-heterocyclic acetamides can find their utility as leading medicinal scaffolds for the treatment of melanogenesis.

4. Experimental

4.1. General

All the chemicals, along with analytical grade solvents, were purchased from Sigma Aldrich, Alfa Aesar (Germany), or Merck through local suppliers. Pre-coated silica gel Al-plates were used for TLC with ethyl acetate and n-hexane as solvent system. Spots were detected by UV254. Gallonkamp apparatus was used to detect melting points in capillary tubes. Elemental analyses were performed on a Foss Heraeus CHN-O-Rapid instrument and were within ±0.4% of the theoretical values. IR spectra (ν cm⁻¹) were recorded by KBr pellet method in the Jasco-320-A spectrophotometer. EI-MS spectra were measured on a JEOL JMS-600H instrument with data processing system. 1H NMR spectra (δ ppm) were recorded at 600MHz (13C NMR spectra, at 150MHz) in DMSO-d₆ using the Bruker Advance III 600 Ascend spectrometer using BBO probe.

4.2. Synthesis of 2-(2-amino-1,3-thiazol-4-yl)acetohydrazide (2)

Ethyl 2-(2-amino-1,3-thiazol-4-yl)acetate (0.15mol.; 1) and 60mL methanol were taken in a 500mL round-bottom flask and hydrazine monohydrate (80%; 20mL) was added into it. The reaction mixture was cooled to 0°C and stirred for 4 h. The solid products were filtered off, washed with ethanol, and dried in a vacuum to give a white solid product 2 (178mg).

Table 3

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<th>No. HBD</th>
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*Abbrevation: WS = Water solubility (log mol/L), IS = intestinal solubility (%abs), SP = Skin permeability (log Kp), BBBP = blood brain barrier permeability (Log BB), CNSP = CNS permeability (LogPS), TC = Total clearance (log ml/min/kg), MTD = Max. tolerat. dose ORAT = Oral Rat Acute Toxicity (LD₅₀), HT = Hepatotoxicity, SS = Skin Sensitization.
stirred for about 2 h at room temperature (RT) and as a result 2-(2-amino-1,3-thiazol-4-yl)acetohydrazide (2) was precipitated out. It was obtained by distilling off the methanol after absolute conversion of reaction. The obtained precipitates of 2 were washed with cold n-hexane and air-dried.

4.3. Synthesis of 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-N-phenyl-1-hydrazinecarbothioamide (4)

2-(2-Amino-1,3-thiazol-4-yl)acetohydrazide (0.13 mol.; 2) was dissolved by heating in methanol (25 mL) taken in 500 mL round flask and after that phenyl isothiocyanate (0.13 mol.; 3) was added. Reaction mixture was kept on refluxing for an hour. After completion of the reaction, precipitates of intermediate compound, 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-N-phenyl-1-hydrazinecarbothioamide (4), were obtained which were filtered and dried to proceed further for cyclization process.

4.4. Synthesis of 5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazole-3-thiol (5)

The intermediate compound, 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-N-phenyl-1-hydrazinecarbothioamide (0.13 mol.; 4), was dissolved in 10% NaOH (100 mL) and the solution was filtered. The filtrate so obtained was acidified with conc. HCl in cold stated to get the precipitates of desired cyclized product 5-[2-(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazole-3-thiol (5). White crystalline solid; Yield: 92%; m.p. 233–234°C; Mol. Formula: C17H11N5S2; Mol. Mass.: 298 gmol⁻¹; IR (KBr, ʋ/cm⁻¹): v 3321 (N≡H str.), 3040 (C≡H str. of aromatic ring), 2912 (eCH2 str.), 1560 (C≡C str. of aromatic ring), 1512 (C≡N str.), 1145 (C≡N–C bond str.), 609 (C≡S str.); 1H NMR (600 MHz, DMSO-d6, δ ppm): δ 13.73 (s, 1H, HS-3′), 7.47–7.46 (m, 3H, H-3′″, H-4′″ & H-5′″), 7.28–7.27 (m, 2H, H-2′″ & H-6′″), 6.88 (s, 2H, H2N-2), 5.85 (s, 1H, H-5), 3.67 (s, 2H, CH2-6); 13C NMR (150 MHz, DMSO-d6, δ ppm): δ 168.78 (C-2), 150.05 (C-5′), 150.58 (C-5″), 144.86 (C-4), 134.17 (C-1″), 129.70 (C-4″), 129.49 (C-3″ & C-5″), 128.63 (C-2″ & C-6″), 103.10 (C-5), 28.60 (C-6). Anal. Calc. for C12H11N5S2 (289.38): C, 49.81; H, 3.83; N, 24.20. Found: C, 49.76; H, 3.79; N, 24.17; EI-MS: m/z 289 [M]+, 256 [C12H11N5S]+, 247 [C11H9N3S2]+, 214 [C11H8N3S]+, 183 [C8H5NS3]+, 138 [C7H6N3S]+, 113 [C6H5NS]+, 77 [C6H3]+, 71 [C5H3S]+.

4.5. General synthesis of 2-[(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazole-3-thiol (5)

5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazole-3-thiol (0.2 g, 5) was dissolved in N,N-dimethyl formamide (DMF, 3 mL) in a 100 mL round bottom flask at room temperature. One pinch of solid

### Table 5

Docking energy values of 9a-n against mushroom tyrosinase.

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Fig. 8. Binding pocket conformation of 9a-n against target protein.

467
LiH was added to this solution and mixture was stirred for half an hour for activation. Then, different electrophiles, 2-bromo-N-(un/substituted-phenyl/benzyl/phenethyl) acetamides (8a–n, one in each reaction), were added in equimolar amount in each respective reaction and the mixture was stirred for 10–12 h. The reaction was monitored by TLC using n-hexane and ethyl acetate solvent system (70:30). A single spot of product on TLC showed the completion of a reaction. The reaction mixture was quenched with excess ice cold distilled water (50 mL). The respective targeted products, 9a–n, were collected through filtration, washed with distilled water and dried for further use.

4.5.1. 2-{{(5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl}sulfanyl}-N-(2,5-dimethylphenyl)acetamide (9a)

Light yellow solid; Yield: 90%; m.p. 102–103 °C; Mol. Formula: C22H22N6OS2; Mol. Mass.: 450 g mol⁻¹; IR (KBr, ν/cm⁻¹): 3343 (N–H str.), 3048 (C–H str. of aromatic ring), 2921 (–CH2 = –), 1665 (C=O str.), 1588 (C=C str. of aromatic ring), 1511 (C=N str.), 1145 (C–N–C bond str.), 612 (C–S str.); 1H NMR (600 MHz, DMSO-d6, δ/ppm): 9.74 (s, 1H, –CO–NH=CH–), 7.54–7.51 (m, 3H, H–3′, H–4′ & H–5′), 7.38–7.36 (m, 2H, H–2′ & H–6′), 7.15 (br.d, J = 7.7, 1H, H–6′′′′), 7.04 (br.t, J = 7.6, 1H, H–5′′′′), 7.00 (br.d, J = 7.3, 1H, H–4′′′′), 6.86 (br.s, 2H, H2N–2), 5.86 (s, 1H, H–5), 4.12 (s, 2H, CH2–2′′′′), 3.79 (s, 2H, CH2–6), 2.23 (s, 3H, CH3–3′′′′), 2.05 (s, 3H, CH3–2′′′′); 13CNMR (150 MHz, DMSO-d6, δ/ppm): 168.12 (C–2), 165.69 (C–1′′′), 153.32 (C–5′), 149.72 (C–3′′), 145.51 (C–4), 136.85 (C–3′′′′), 135.61 (C–1′′′′), 132.82 (C–1′′), 130.66 (C–2′′′′), 129.71 (C–4′′), 129.48 (C–3′ & C–5′), 127.07 (C–2′′ & C–6′′), 126.85 (C–4′′′′), 125.06 (C–5′′′′), 122.88 (C–6′′′′), 102.09 (C–5′), 36.12 (C–2′′′′), 27.39 (C–6), 20.01 (CH3–3′′′′), 13.77 (CH3–2′′′′). Anal. Calc. for C22H22N6OS2 (450.58): C, 58.64; H, 4.92; N, 18.65, Found: C, 58.71; H, 4.97; N, 18.72. El–MS (m/z): 450 [M]+, 330 [C14H12N5OS2]+, 270 [C13H10N4OS]+, 256 [C14H10NS]+, 148 [C4H10NO]+, 120 [C6H10N]+, 71 [C3H5S]+.

4.5.2. 2-{{(5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl}-N-(2,5-dimethylphenyl)acetamide (9b)

Light orange solid; Yield: 89%; m.p. 172–173 °C; Mol. Formula: C22H22N6OS2; Mol. Mass.: 450 g mol⁻¹; IR (KBr, ν/cm⁻¹): 3342 (N–H str.), 3052 (C–H str. of aromatic ring), 2918 (–CH2 = –), 1670 (C=O str.), 1577 (C=C str. of aromatic ring), 1519 (C=N str.), 1152 (C–N–C bond str.), 613 (C–S str.); 1H NMR (600 MHz, DMSO-d6, δ/ppm): 9.62 (s, 1H, –CO–NH=CH–), 7.53–7.51 (m, 3H, H–3′, H–4′ & H–5′), 7.37–7.36 (m, 2H, H–2′ & H–6′), 7.28 (br.d, J = 8.1, 1H, H–6′′′), 7.01 (br.s, 1H, H–3′′′′), 6.95 (br.d, J = 8.1, 1H, H–5′′′′), 6.85 (br.s, 2H, H2N–2), 5.86 (s, 1H, H–5), 4.11 (s, 2H, CH2–2′′′′), 3.78 (s, 2H, CH2–6), 2.23 (s, 3H, CH3–2′′′′), 2.14 (s, 3H, CH3–2′′′′); 13CNMR (150 MHz, DMSO-d6, δ/ppm): 168.12 (C–2), 165.69 (C–1′′′), 156.12 (C–5′), 153.42 (C–3′′), 149.84 (C–3′), 145.69 (C–4), 134.24 (C–1′′′′), 133.37 (C–2′′′′), 132.89 (C–1′′), 131.16 (C–4′′′′), 130.79 (C–5′′′′), 129.80 (C–4′′), 129.56 (C–3′ & C–5′), 127.15 (C–2′′ & C–6′′), 126.41 (C–3′′′′), 124.36 (C–6′′′′), 102.18 (C–5′), 36.24 (C–2′′′′), 27.47 (C–6), 20.42 (CH3–4′′′′), 17.65 (CH3–2′′′′). Anal. Calc. for C22H22N6OS2 (450.58): C, 58.64; H, 4.92; N, 18.65, Found: C, 58.70; H, 4.94; N, 18.66. El–MS (m/z): 450 [M]+, 330 [C14H12N5OS2]+, 270 [C13H10N4OS]+, 256 [C14H10NS]+, 148 [C4H10NO]+, 120 [C6H10N]+, 71 [C3H5S]+.

Fig. 9. Docking complexes of 9g. The ligand structures 9g is highlighted in purple color while the interactive residues are depicted in red color. The target protein is justified in different colors in ribbon format.
δ/ppm): 168.13 (C-2), 165.62 (C-1′′′), 153.48 (C-5′), 149.85 (C-3′), 145.40 (C-4), 135.66 (C-1’′′′), 134.86 (C-2′′′), 132.80 (C-1′′), 129.98 (C-4′′′), 129.73 (C-4′′), 129.59 (C-3′′′′), 129.49 (C-3′′ & C-5′′), 127.07 (C-2′′ & C-6′′), 124.68 (C-6′′′′), 102.09 (C-5), 36.17 (C-2′′′), 27.40 (C-6), 21.11 (CH3-5′′′′), 16.81 (CH3-2′′′′).


4.5.4. 2-({5-[{(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl}sulfanyl}-N-(2,6-dimethylphenyl)acetamide (9d)

Light yellow solid; Yield: 88%; m.p. 166–167°C; Mol. Formula: C22H22N6OS2; Mol. Mass.: 450 gmol−1; IR (KBr, ʋ/cm−1): 3359 (N–H str.),3062 (C–H str.of aromatic ring),2925 (C–H str.),1676 (C=O str.), 1573 (C=C str of aromatic ring), 1531 (C=C str of aromatic ring), 1157 (C–N–C bond str.), 618 (C–N str); 1H NMR (600 MHz, DMSO-d6, δ/ppm): 9.62 (1H, –CO–NH-1′′′′), 7.54–7.52 (3H, H-3′′, H-4′′ & H-5′′), 7.38–7.37 (3H, H-4′′′′ & H-5′′′′), 6.85 (br.s, 2H, H-N=2), 5.85 (1H, H-5′), 4.13 (2H, CH2-2′′′), 3.79 (2H, CH2-6), 2.10 (2H, CH2-4′′′′ & CH2-5′′′′); 13C NMR (150 MHz, DMSO-d6, δ/ppm): 168.19 (C-2), 165.30 (C-1′′′), 153.33 (C-5′), 149.73 (C-3′), 145.64 (C-4), 135.13 (C-2′′′′ & C-6′′′′), 134.62 (C-1′′′′), 132.92 (C-1′′′′), 129.79 (C-4′′′), 129.57 (C-3′′ & C-5′′), 127.59 (C-2′′′′ & C-6′′′′), 124.71 (C-3′′′′ & C-5′′′′), 115.7 (C-2′′′′ & C-6′′′′), 102.09 (C-5), 35.67 (C-2′′′), 27.40 (C-6), 21.11 (CH3-5′′′′), 16.81 (CH3-2′′′′). Anal. Calc. for C22H22N6OS2 (450.58): C,58.64; H,4.92; N,18.65. Found: C,58.77; H,4.82; N, 18.69; EI-MS (m/z): 450 [M]+, 330 [C14H12N5OS2]+, 270 [C13H10N4OS]+, 256 [C12H10N5S]+, 148 [C 9H10NO]+, 120 [C8H10N]+, 71 [C3H3S]+.

4.5.5. 2-({5-[{(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl}sulfanyl}-N-(3,4-dimethylphenyl)acetamide (9e)

Bright yellow solid; Yield: 91%; m.p. 120–121°C; Mol. Formula: C22H22N6OS2; Mol. Mass.: 450 gmol−1; IR (KBr, ʋ/cm−1): 3338 (N–H str.),3064 (C–H str.of aromatic ring),2921 (C–H str.), 1667 (C=O str.), 1573 (C=C str of aromatic ring), 1531 (C=C str of aromatic ring), 1157 (C–N–C bond str.), 632 (C–N str); 1H NMR (600 MHz, DMSO-d6, δ/ppm): 10.16 (1H, –CO–NH-1′′′′), 7.52–7.50 (3H, H-3′′, H-4′′ & H-5′′), 7.37–7.35 (2H, H-2′′ & H-6′′), 7.32 (1H, H-2′′′′), 7.26–7.25 (1H, H-6′′′′), 6.87 (2H, H-N=2), 5.85 (1H, H-5′), 4.10 (2H, CH2-2′′′′), 3.76 (2H, CH2-6), 2.18 (3H, CH2-3′′′′); 13C NMR (150 MHz, DMSO-d6, δ/ppm): 168.21 (C-2), 165.18 (C-1′′′′), 153.36 (C-5′), 149.72 (C-3′′), 145.51 (C-4), 136.47 (C-3′′′′), 136.32 (C-1′′′′), 132.88 (C-1′′), 131.21 (C-2′′′′), 129.77 (C-4′′′), 129.54 (C-3′′ & C-5′′), 127.13 (C-2′′′′ & C-6′′′′),
5.4. 2-((2-amino-1,3-thiazol-4-yl)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl-N-(5,5-dimethylphenyl)acetamide (9f)

Light yellow solid; Yield: 88%; m.p. 110–111 °C; Mol. Formula: C22H22N6O2S2; Mol. Mass.: 466 g/mol; IR (KBr, v/cm−1): 3348 (N–H str.), 3067 (C–H str. of aromatic ring), 2972 (C–H str.), 1654 (C=O str.), 1556 (C–C str. of aromatic ring), 1552 (C=O str.), 1161 (C–O str.), 628 (C–H str.); 13C NMR (150 MHz, DMSO-d6, δ/ppm): 168.21 (C-2), 165.79 (C-1′′′), 153.40 (C-5′), 149.78 (C-3′′′), 145.58 (C-4′′′), 138.96 (C-1′′′′), 138.42 (C-1′′), 130.29 (C-3′′), 129.81 (C-4′′′′), 129.06 (C-3′′′′ & C-5′′), 127.15 (C-2′′′ & C-6′′′), 125.94 (C-5′′′′), 125.23 (C-4′′′′), 124.27 (C-6′′′′), 102.18 (C-5′′), 36.25 (C-2′′′), 27.47 (C-6), 17.11 (CH3-S), 71 [C6H5-S].

5.5. 2-((2-amino-1,3-thiazol-4-yl)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl-N-(2-ethylphenyl)acetamide (9g)

Light orange solid; Yield: 91%; m.p.: 144–145 °C; Mol. Formula: C22H22N6O2S2; Mol. Mass.: 430 g/mol; IR (KBr, v/cm−1): 3346 (N–H str.), 3067 (C–H str. of aromatic ring), 2972 (C–H str.), 1654 (C=O str.), 1556 (C–C str. of aromatic ring), 1552 (C=O str.), 1161 (C–O str.), 628 (C–H str.); 13C NMR (150 MHz, DMSO-d6, δ/ppm): 168.12 (C-2), 165.79 (C-1′′′), 153.33 (C-5′), 149.78 (C-3′′′), 145.58 (C-4′′′), 138.96 (C-1′′′′), 138.42 (C-1′′), 130.29 (C-3′′), 129.81 (C-4′′′′), 129.06 (C-3′′′′ & C-5′′), 127.15 (C-2′′′ & C-6′′′), 125.94 (C-5′′′′), 125.23 (C-4′′′′), 124.27 (C-6′′′′), 102.18 (C-5′′), 36.25 (C-2′′′), 27.47 (C-6), 17.11 (CH3-S), 71 [C6H5-S].

5.6. 2-((2-amino-1,3-thiazol-4-yl)methyl)-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl-N-(5,5-dimethylphenyl)acetamide (9h)

Brick red solid; Yield: 92%; m.p.: 98–99 °C; Mol. Formula: C22H22N6O2S2; Mol. Mass.: 450 g/mol; IR (KBr, v/cm−1): 3365 (N–H str.), 3045 (C–H str. of aromatic ring), 2915 (C–H str.), 1658 (C=O str.), 1582 (C–C str. of aromatic ring), 1519 (C=N str.), 1152 (C=N–C bond str.), 611 (C=O str.); 1H NMR (600 MHz, DMSO-d6, δ/ppm): 9.69 (s, 1H, –CO–NH–), 7.53–7.51 (m, 3H, H–3′′, H–4′′ & H–5′′), 7.40–7.36 (m, 2H, H–2′′ & H–6′′), 7.27 (br, d, J = 7.3, 1H, H–5′′′′), 7.17–7.13 (m, 2H, H–4′′′′ & H–5′′′′), 6.85 (brs, 2H, H-N2), 5.86 (s, 1H, H–5), 4.13 (s, 2H, CH2–2′′′′), 3.79 (s, 2H, CH2–6), 2.57 (q, J = 7.5, 2H, CH2–2′′), 1.09 (t, J = 7.5, 3H, CH3–2′′), 13C NMR (150 MHz, DMSO-d6, δ/ppm): 168.12 (C-2), 165.99 (C-1′′′), 153.35 (C-5′), 149.79 (C-3′′), 145.52 (C-4′′′), 137.24 (C-1′′′′), 135.14 (C-3′′′′), 132.79 (C-1′′), 129.72 (C-4′′), 129.49 (C-3′′′ & C-5′′), 128.45 (C-3′′′′), 127.06 (C-2′′′ & C-6′′′), 125.82 (C-5′′′′), 125.54 (C-4′′′′), 125.12 (C-6′′′′) 100.08 (C-5′′), 36.12 (C-2′′′), 27.38 (C-6), 23.58 (CH3–CH2–2′′′′), 14.11 (CH3–CH2–3′′′′).
\[(CH_2=CH-C=O)\] = 36.61 (C-2′′′), 27.39 (C-6), 14.56 (CH_2=CH-C=O)\] \\
Anal. Calc. for C_{22}H_{22}N_6O_2S (466.58): C, 56.63; H, 4.75; N, 18.01. \\
Found: C, 56.65; H, 4.90; N, 18.17; El-MS (m/z): 466 [M]+, 530 [C_{14}H_{12}N_5O_2S]+, 270 [C_{14}H_{12}N_5O_2S]+, 256 [C_{12}H_{10}N_5S]+, 189 [C_{14}H_{10}N_5S]+, 136 [C_{14}H_{10}N_5S]+, 113 [C_{14}H_{10}N_5S]+, 107 [C_{14}H_{10}N_5S]+, 77 [C_{14}H_{10}N_5S]+.

4.5.12. 2-((5-[(2-aminio-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl)-N-benzylacetamide (9)

Dark orange solid; Yield: 90%; m.p. 128–139 °C; Mol. Formula: C_{22}H_{22}N_{6}O_{2}S; Mol. Mass.: 436 g mol\(^{-1}\); IR (KBr, cm\(^{-1}\)): 3349 (N-OH str.), 1682 (C=O str.), 1538 (C=C str.), 1527 (C=C str.), 1497 (C=C str.), 1417 (C=C str.), 1394 (C=N str.), 1358 (C=C str.), 1297 (C=C str.), 1287 (C=C str.)

4.5.13. 2-((5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl)-N-phenylacetamide (9m)

Light yellow solid; Yield: 92%; m.p. 158–159 °C; Mol. Formula: C_{22}H_{22}N_{6}O_{2}S; Mol. Mass.: 436 g mol\(^{-1}\); IR (KBr, cm\(^{-1}\)): 3349 (N-OH str.), 1682 (C=O str.), 1538 (C=C str.), 1527 (C=C str.), 1497 (C=C str.), 1417 (C=C str.), 1394 (C=N str.), 1358 (C=C str.), 1297 (C=C str.), 1287 (C=C str.)

4.5.14. 2-((5-[(2-aminio-1,3-thiazol-4-yl)methyl]-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl)-N-benzylacetamide (9m)

Drak brown gummy solid; Yield: 82%; Mol. Formula: C_{22}H_{22}N_{6}O_{2}S; Mol. Mass.: 450 g mol\(^{-1}\); IR (KBr, cm\(^{-1}\)): 3349 (N-OH str.), 1682 (C=O str.), 1538 (C=C str.), 1527 (C=C str.), 1497 (C=C str.), 1417 (C=C str.), 1394 (C=N str.), 1358 (C=C str.), 1297 (C=C str.), 1287 (C=C str.)

4.6. Mushroom tyrosinase inhibition assay

The mushroom tyrosinase (Sigma Chemical, USA) inhibition was performed following our previously reported methods [58,59]. In detail, 140 µL of phosphate buffer (20 mM, pH 6.8), 20 µL of mushroom tyrosinase (30 U/mL) and 20 µL of the inhibitor solution were placed in the wells of a 96-well microplate. After pre-incubation for 10 min at room temperature, 20 µL of L-DOPA (3,4-dihydroxyphenylalanine, Sigma Chemical, USA) (0.85 mM) was added and the assay plate was further incubated at 25 °C for 20 min. Afterward, the absorbance of dopamine was measured at 475 nm using a microplate reader (OPTI Max, Tunable). Kojic acid was used as a reference inhibitor and phosphatase buffer was used as a negative control. The amount of inhibition by the test compounds was expressed as the percentage of concentration necessary to achieve 50% inhibition (IC_{50}). Each concentration was analyzed in three independent experiments. IC_{50} values were calculated by nonlinear regression using GraphPad Prism 5.0.

The % Inhibition of tyrosinase was calculated as following: 
\[
\text{Inhibition(\%)} = \left(\frac{\text{Absorbance of Sample} - \text{Absorbance of Negative Control}}{\text{Absorbance of Positive Control}}\right) \times 100
\]

4.7. Kinetic analysis of the inhibition of tyrosinase

On the basis of IC_{50}, we selected the most potent 9g compound for kinetic analysis. A series of experiments were performed to determine the inhibition kinetics of 9g by following the already reported methods [60,61]. The inhibitor concentrations for 9g were 0.00, 0.0077, and 0.0155 µM. Substrate L-DOPA concentrations were between 0.0625 and 2 mM in all kinetic studies. Pre-incubation and measurement time was the same as discussed in the mushroom tyrosinase inhibition assay protocol. Maximal initial velocity was determined from the initial linear portion of absorbance up to five minutes after addition of enzyme at a 30 s interval. The inhibition type of the enzyme was assayed by Line-weaver–Burk plots of the inverse of velocities (1/V) versus the inverse of substrate concentration 1/(L-DOPA) μM\(^{-1}\). The El dissociation constant K_{i} was determined by the secondary plot of 1/V versus inhibitors concentrations.

4.8. Hemolytic activity

Bovine blood sample was collected in EDTA that was diluted with saline (0.9% NaCl), and centrifuged at 10000xg for 10 min. The erythrocytes separated diluted in phosphate buffer saline of pH 7.4 and a suspension was made. Add 20 µL of synthetic compounds solution (10 mg/mL) in 180 µL of RBCs suspension and incubate for 30 min at room temperature. PBS was used as negative control and Triton 100-X was taken as positive control [62,63]. The %age of hemolysis was taken as by using formula:

\[
\text{(% of Hemolysis)} = \left(\frac{\text{Absorbance of Sample} - \text{Absorbance of Negative Control}}{\text{Absorbance of Positive Control}}\right) \times 100
\]

4.9. in silico methodology

4.9.1. Retrieval and preparation of mushroom tyrosinase protein in schrodinger suite

The mushroom tyrosinase protein structure was retrieved from Protein Data Bank (PDB) (www.rcsb.org) with PDBID 2Y9X in protein preparation wizard. The selected crystal structure of mushroom tyrosinase was preprocessed and minimized using default parameters in
Maestro interface.

4.9.2. Grid generation and molecular docking
Prior to molecular docking, the optimized mushroom tyrosinase structure was prepared using the "Protein Preparation Wizard" workflow in Schrödinger Suite. Bond orders were assigned and hydrogen atoms were added to the protein. The structure was then minimized to reach the converged root mean square deviation (RMSD) of 0.30 Å with the OPLS 2005 force field. The active site of the enzyme is defined from the co-crystallized ligands from Protein Data Bank and literature data.[53,54] Furthermore, docking experiment was performed against all synthesized ligands and target protein by using Glide docking protocol.[64] The predicted binding energies (docking scores) and conformational positions of ligands within active region of protein were also analyzed using Glide experiment. Throughout the docking simulations, both partial flexibility and full flexibility around the active site residues were sketched in ACD/ChemSketch.[45] Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.036.

References