Synthesis, *in vitro* urease inhibitory potential and molecular docking study of Benzimidazole analogues

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ABSTRACT

Despite of many diverse biological activities exhibited by benzimidazole scaffold, it is rarely explored for the urease inhibitory potential. For that purpose, benzimidazole analogues **1–19** were synthesized and screened for *in vitro* urease inhibitory potential. Structures of all synthetic analogues were deduced by different spectroscopic techniques. All analogues revealed inhibition potential with IC_{50} values of 0.90 ± 0.01 to $35.20 \pm 1.10\,\mu M$, when compared with the standard thiourea ($IC_{50} = 21.40 \pm 0.21\,\mu M$). Limited SAR suggested that the variations in the inhibitory potentials of the analogues are the result of different substitutions on phenyl ring. In order to rationalize the binding interactions of most active compounds with the active site of urease enzyme, molecular docking study was conducted.

1. Introduction

Urease is a nickel-containing metalloenzyme that catalyzes the conversion of urea into ammonia and carbamate in micro-organism and various plant species [1–5]. The hydrolysis of urea by urease enzyme occurs speedily at 10^{14} times faster than the hydrolysis reaction by uncatalyzed way. This reaction caused by urease enzyme results in sudden increase of the overall pH, which causes negative effects to agriculture production, animal health and human beings. Urease enzyme has revealed to be significant lethal factor to cause hepatic coma, urinary catheter encrustation, pyelonephritis and infection stones in human beings and animals [6,7]. Further, urease enzyme can harshly decline the efficacy of urea fertilizers to cause the discharge of large quantities of ammonia and also induce damage of plant by ammonia toxicity and pH rise in soil [8]. In this context, urease enzyme inhibition study has attracted growing attention to tackle the negative effects

[9–11]. Moreover, urease inhibitory studies are important in elucidating the catalytic mechanism of urease which is still unclear as has been extensively discussed in a recent article [12]. However, the efficiency of currently available inhibitors is low and the complete potential of urease inhibition has not yet been discovered [13]. Consequently, the ability to control the degree of enzymatic urea hydrolysis by urease enzyme inhibitors is significant goal to chase [14].

Nitrogen-containing heterocycles have attracted considerable attention due to their wide occurrence and pharmacological importance. Benzimidazole is an aromatic heterocyclic organic compound having benzene ring fused with an imidazole ring. Imidazole ring is the part of many natural products including purine, histamine, histidine, and nucleic acid. Due to its polar and ionisable ability, it proves to be characteristics pharmacokinetic for lead molecules by enhancing their solubility [15–17]. Benzimidazole is one of the privileged structures in medicinal chemistry due to its wide range of activities including

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analgesic, antidiabetic, anti-inflammatory, antiulcer, antifungal, antimicrobial, anticancer, antiprotozoal and antiviral activities *etc* [18–21].

Our research group has been working on design and synthesis of heterocyclic compounds in search of potential lead compounds since many years and had found promising results [22–29]. We have compared our synthesized analogues with already reported benzimidazole analogues by Emre Mentese et al., for anti-urease activity [30,31]. We have also reported benzimidazole derivatives as *α*-glucosidase inhibitors [32,33] and 2-(2-Pyridyl) benzimidazole derivatives as potent inhibitors for urease enzyme [34] but there is still need to explore more compounds for this activity in order to identify lead candidates for more advances research in future. Thus, we decided to screen a library of substituted benzimidazoles for urease inhibitory activity.

2. Results and discussion

2.1. Chemistry

We mixed 1*H*-benzimidazole-2-thiol **(I)** (1 mmol) with methyl 4-(bromomethyl) benzoate **(II)** (1 mmol) in acetone in the presence of K_2CO_3 and reflux for 5 hrs to give methyl 4-(((1*H*-benzimidazol-2-yl) thio)methyl)benzoate **(III)** as intermediate product. The intermediate product **(III)** was further treated with hydrazine hydrate in ethanol and reflux for 6 hrs to give 4-(((1*H*-benzimidazol-2-yl)thio)methyl)benzohydrazide **(IV)** as second intermediate product. The intermediate product **(IV)** was finally treated and refluxed with different substituted aldehyde/acetophenone to give the desired products **(1–19)**. Completion of reaction was monitored by TLC. After completion of reaction; the product was filtered, washed with *n*-hexane and dried (see Table 1).

2.2. In vitro urease inhibitory potential

We have synthesized nineteen benzimidazole analogues **(1–19)** which have varied degree of urease inhibition ranging in between 0.90 ± 0.01and 35.20 ± 1.10 μM when compared with the standard drug thiourea having IC_{50} value 21.40 ± 0.21 μM. All analogues showed excellent urease inhibitory potentials. The structure-activity relationship (SAR) was mainly based upon by bring about difference of substituents on phenyl ring.

If we compare analogue **1** (IC_{50} = 4.60 ± 0.2 μM) with analogue **3** (IC_{50} = 15.20 ± 0.5 μM) and analogue **19** (IC_{50} = 14.80 ± 0.30 μM), all three analogues have nitro group on phenyl ring, but the arrangement of nitro group is different in them which confirm that the difference in position of substituents greatly affect the inhibitory potentials of the analogues.

If we compare analogue **2** (IC_{50} = 8.60 ± 0.3 μM) having one chloro group at 4-position on phenyl ring with analogue **4** (IC_{50} = 3.20 ± 0.01 μM) having two chloro groups at 2,4-position on phenyl ring and analogue **9** (IC_{50} = 5.10 ± 0.2 μM) also having two chloro groups at 3,4-position on phenyl ring. All compounds have the same chloro groups but the position as well as number of the chloro group is different at the phenyl ring. Compound **4** was found to be superior who showed that position as well as number of substituent also play role in this inhibition.

By comparing analogue **6** (IC_{50} = 1.40 ± 0.01 μM) having one methoxy and one hydroxy substituents with analogue **14** (IC_{50} = 1.90 ± 0.01 μM) also having one methoxy and one hydroxy substituents on phenyl ring. In analogue **6,** the methoxy group is present at 4-position and hydroxy is present at 3-position and in analogue **14,** the methoxy group is present at 3-position and hydroxy at 4-position on phenyl ring. Both analogues have the same methoxy and hydroxy groups but the position of the hydroxy and methoxy groups are different on phenyl ring. Compound **6** was found to be superior who showed that position of substituent also play role in this inhibition.

In this study, we observed that either electron withdrawing group (EWG) or electron donating group (EDG) on phenyl ring showed potential but the slight difference in potential was mainly affected by the position of the substituent as well as in some cases the number of substituent also play a role. To understand the binding interaction of the most active analogs molecular docking study was performed.
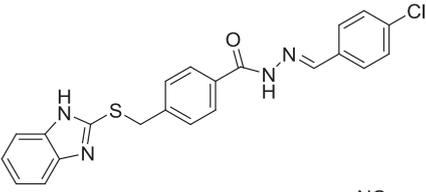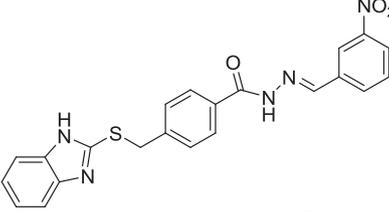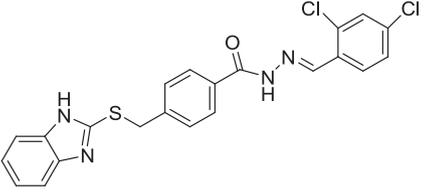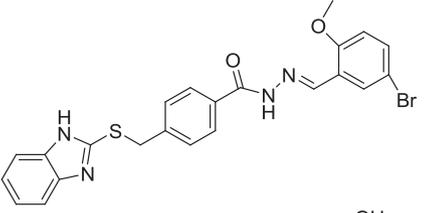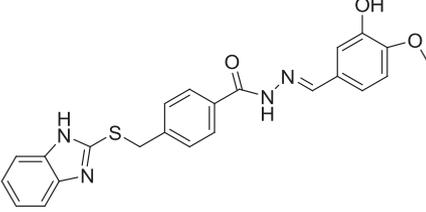
2.3. Molecular docking

Molecular Operating Environment (MOE) package [35] was used to perform molecular docking study to predict the binding mode of the synthesized compounds within the active site of urease enzyme. First, the 3D structures of the synthesized derivatives were generated by using the builder tool executed in MOE package. Next, all the compounds were subjected for protonation, and energy minimized using the default parameters of the MOE (gradient: 0.05, Force Field: MMFF94X) and saved in mdb (Molecular knowledge Base) file format. The 3D structure of the target protein retrieved from the protein databank (PDB ID 4UBP). The retrieved protein then opened in MOE package, all the water molecules removed, later on, 3D protonation carried. After 3D protonation, the protein was energy minimized to get a stable conformation of the protein using the default parameters of the MOE package. For docking studies, the default parameters of the MOE package were used, i.e., Placement: Triangle Matcher, rescoring 1: London dG, Refinement: Force field, Rescoring 2: GBVI/WSA. For each ligand ten conformations could be fashioned, and the top-ranked conformations based on docking score were selected for additional analysis (see Fig. 1 and Scheme 1).

2.4. Molecular docking study

Molecular docking study was carried out through MOE package, in order to illustrate the binding mode of interaction of the entire synthesized compound enlisted in the current study (**1–19**), again the urease enzyme to validate the experimental results further. The primarily favorable docking conformations were ascertained within the active site with proper orientation for all the compounds. Generally, the ligand binding site of the corresponding enzyme comprises from both hydrophobic and hydrophilic amino acids. A bunch of total five surface residues (A170, 366, K169, L319, and C322) makes the hydrophobic region, while the hydrophilic region, totally nine essential residues G166, 223, R339, D224, 494, H315, 323, 324, and 249 respectively as shown in Fig. 2A. The two embedded Ni ions (Ni198 & 799) conjointly participate a significant role by linking the critical amino acid and ligands. However, from the molecular docking study, it has been observed that all the compounds fit well in the active pocket of the urease enzyme. More ever, the most promising docked conformation of each compound was evaluated further for binding mode analysis, based on the scores from the GBVI/WSA binding free energy calculation. Generally, from the post-docking analysis, it was observed that the enlisted synthesized compound possesses different substituted groups, like some, have electron withdrawing (EW), and other have donating (ED) groups, and hence, the position and the changing groups from EW to ED or vice versa ultimately altered the enzyme activity. The high potency for most active compound **18** (0.90 ± 0.01) was confirmed from both the fit-well behavior and ligand–protein interaction (LPI) analysis, that this compound found the only one in the series which adopts most favorable interactions with the essential active site residues, include R369, 339, L319, T362, D363, additionally the two embedded Ni ions were found in conjugate with; Ni799-O[19] and Ni-798-O[30] of the candidate compound.
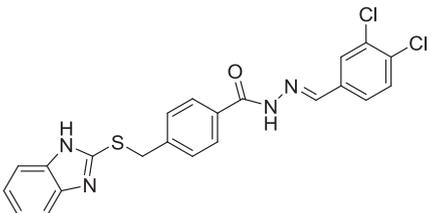
Furthermore, this Ni799 was observed in ionic coordination with the modified residue KCX220. The high LPI profile observed for **18** (Fig. 2B), further demonstrate that the high potency of the candidate compound might be due to the attached EDG (di-hydroxyl). The −OH group has a more activating effect on some positions; ortho & para around the ring than others means that incoming groups will go into

Table 1
Different analogues of benzimidazole and their urease inhibitory activity.
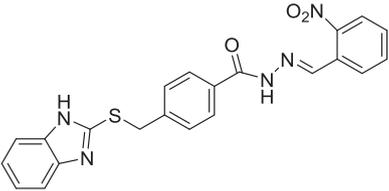
Code	Structure	IC$_{50}$
1		4.60 ± 0.2
2		8.60 ± 0.3
3		15.20 ± 0.5
4		3.20 ± 0.01
5		13.10 ± 0.3
6		1.40 ± 0.01
7		18.6 ± 0.50
8		15.70 ± 0.50
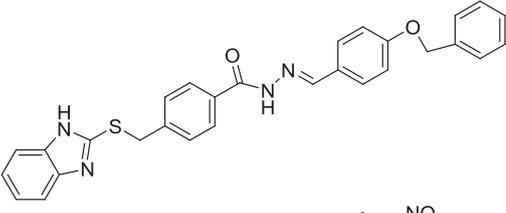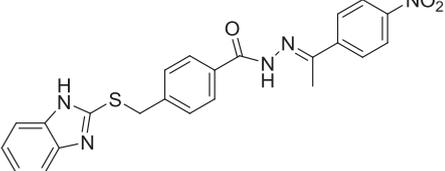
Table 1 (*continued*)

Code	Structure	IC$_{50}$
9		5.10 ± 0.2
10		1.20 ± 0.01
11		35.20 ± 1.10
12		31.20 ± 1.10
13		13.60 ± 0.40
14		1.90 ± 0.01
15		7.10 ± 0.20
16		6.6 ± 0.20
17		8.20 ± 0.20
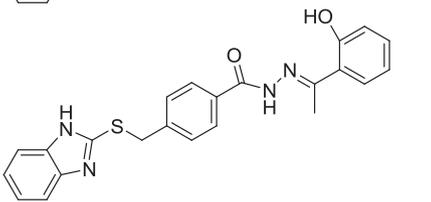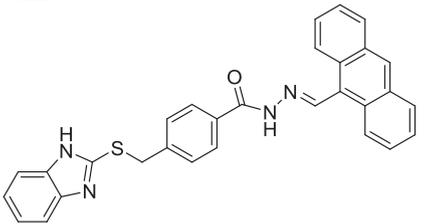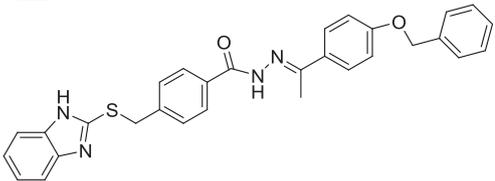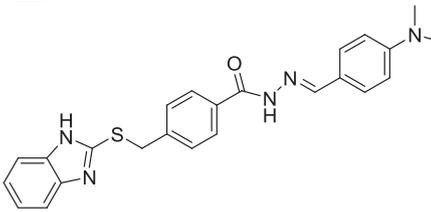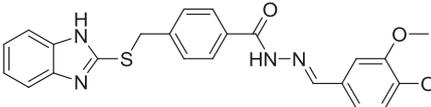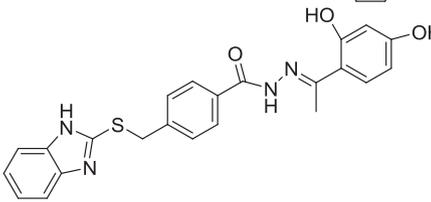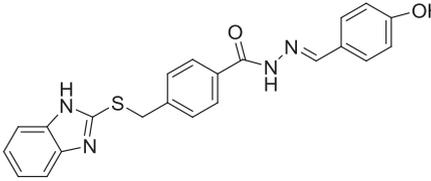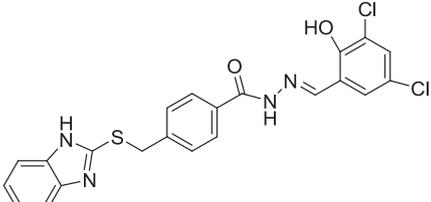
Table 1 (*continued*)

Code	Structure	IC$_{50}$
18		0.90 ± 0.01
19		14.80 ± 0.30
	Thiourea	21.40 ± 0.21 μM

some positions much faster than they will into others, and hence enhance the enzyme activity. As the variation occurrence from di- to mono or reverse, as well the position from -ortho to -para or reveres ultimately enhance or reduce the enzyme activity. The change as mentioned above was observed in the case of second rank compound **10**

(1.20 ± 0.01). The LPI profile for compound **10** indicates that this compound adopts favorable interaction with the essential residue like; R369, D363, H249, and KCX220 (Fig. 2C).

Additionally, the Ni798 has observed ion ionic interaction with the S of the candidate compound. The low potency in comparison with the
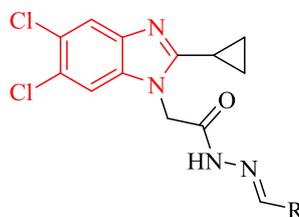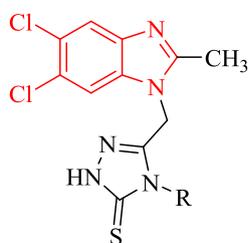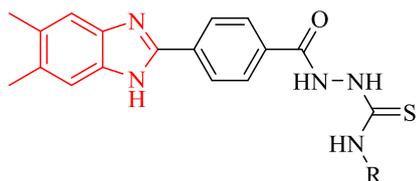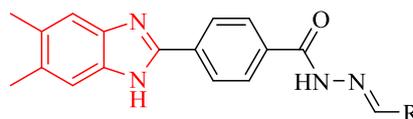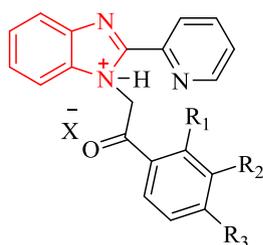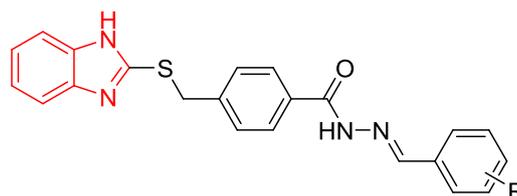
Fig. 1. Benzimidazole based already identified inhibitors of *α*-glucosidase and urease enzymes and newly synthesized derivatives as urease inhibitors **1–19**.
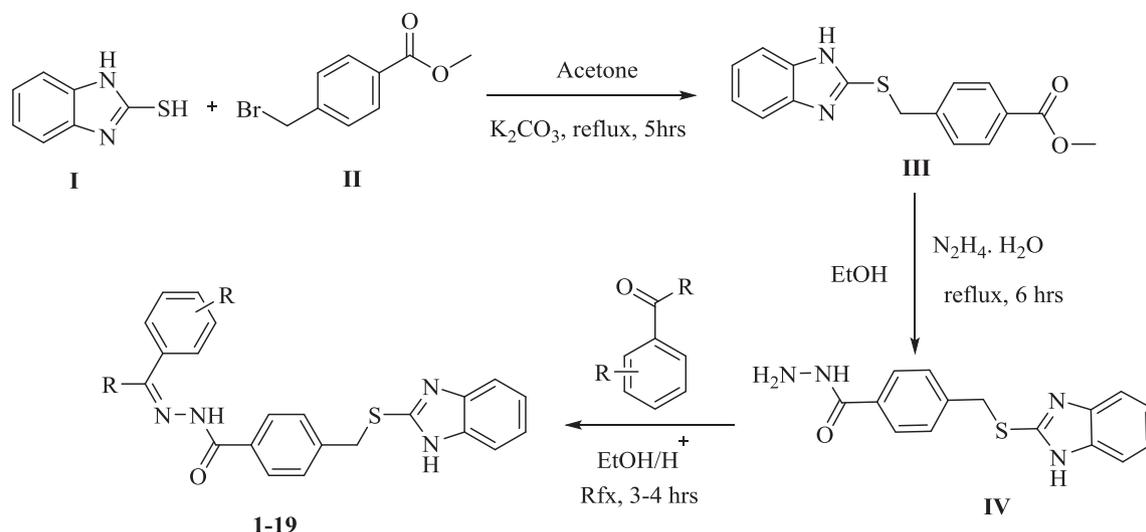
Scheme 1. Synthesis of benzimidazole analogues (**1–19**).

highly potent compound **18** might be due to the attached EDG (mono-OH)-group at benzene ring. Both the compounds possess the same EDG but hold differences only on their benzene ring attached group (di-OH and mono-OH). More ever, the same LPI profile was observed for compound **6** (Fig. 2D) and **14** (Fig. 2E), but less than the other most active compound. The compound **4**, hold the EWG, showed less potency, and LPI profile again Urease enzyme, like an adopted interaction with H222, 249, and D363 (Fig. 2F). The high potency of this compound might be due to EWG attached on benzene, hence, withdraw the electron from the benzene and remain the benzene partial +ve, so further this benzene rings unable to form pi-interaction.

The docking pose of almost all potent compounds computationally inhibited the catalytic activities of the urease by binding determinedly through strong hydrogen bonding, hydrophobic, and polar interactions with key residues, additionally, the compounds hold the EDG showed high potency as compared to the compounds possess the EWG.

2.5. ADMET analysis

ADMET pharmacokinetics is a very important method in the designing and screening of drug, which is responsible for failure of drug. The ADMET properties of the drug candidates are greatly influenced by the optimum value of the molecular weight, *LogP*, hydrogen bond donor, hydrogen bond acceptor, intestinal absorption, water solubility, blood brain barrier (BBB) penetration level. We used Lipinski's rule of five to determine the drug-likeness of these compounds. By this rule, most orally administered drugs have molecular weight less than 500, a distribution ratio less than 5, less than 5 hydrogen bond donors and less than 10 hydrogen bond acceptors. The ADMET properties of these newly synthesized compounds **1–19** was measured using online server PKCM (biosig.unimelb.edu.au/pkcsm/prediction) as a drug reference was reported in Table 2. From the Table 2, it is clear that all our synthesized compounds obeyed the Lipinski's rule of 5. These results showed that our compounds are drug-like compounds. Furthermore, it was also observed that these compounds have the capability to cross the
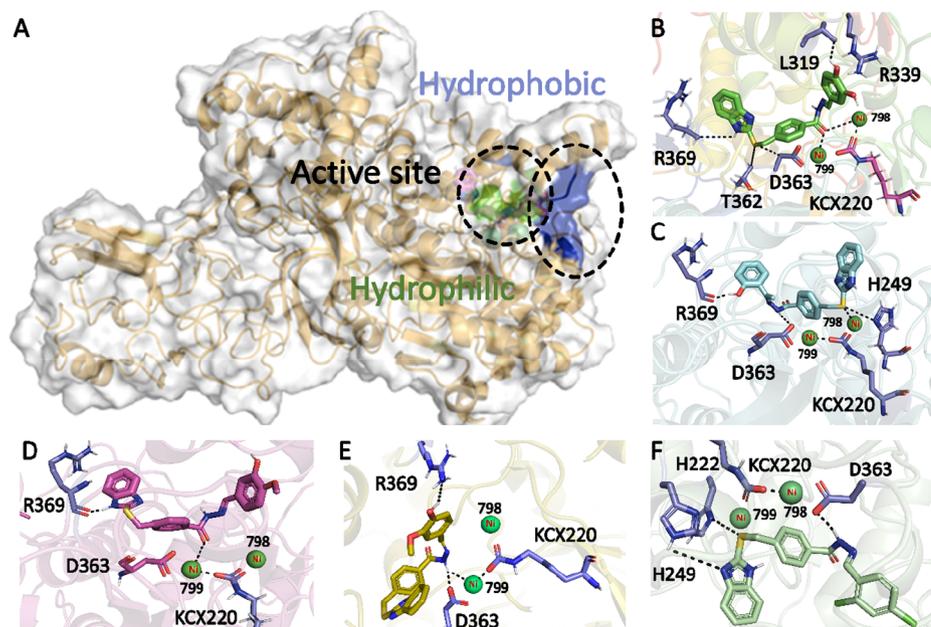
Fig. 2. The ligand–protein interaction (LPI) profile for synthesized compounds against urease enzyme. (A) The surface representation of the urease enzyme (PDB ID 4UBP). The hydrophobic and hydrophilic regions were colored to green and blue, additionally, the two Nickle ions (798 & 799 were shown in light green spheres. (B) The ligand-protein interaction poses for the most active compound **18**, (C) for **10**, (D) for **6**, (E) **14**, and (F) **4**.

Table 2
Prediction of ADMET properties of compounds **1–19**.

Compound	MW	LogP	H bond acceptor	H bond donor	Solubility	Absorption	BBB
1	431.477	4.5273	6	2	−2.928	89.971	−0.876
2	420.925	5.2725	4	2	−2.499	77.499	−0.865
3	431.477	4.5273	6	2	−2.929	89.991	−0.887
4	455.37	5.9259	4	2	−2.931	76.71	−1.037
5	495.402	5.3902	5	2	−2.988	78.536	−1.057
6	432.505	4.3333	6	3	−2.963	87.313	−0.974
7	492.604	6.1981	5	2	−2.922	78.372	−0.882
8	445.504	4.9174	6	2	−2.965	91.697	−0.9
9	455.37	5.9259	4	2	−2.931	77.215	−1.032
10	416.506	4.7148	5	3	−2.956	77.762	−1.016
11	486.6	6.9255	4	2	−2.896	80.291	−0.733
12	506.631	6.5882	5	2	−2.925	78.371	−0.892
13	429.549	4.6851	5	2	−2.988	80.577	−0.72
14	432.505	4.3333	6	3	−2.958	84.256	−0.965
15	432.505	4.4204	6	4	−2.925	83.458	−1.29
16	402.479	4.3247	5	3	−2.916	76.431	−0.898
17	471.369	5.6315	5	3	−2.917	76.802	−1.224
18	418.478	4.0303	6	4	−2.9	78.984	−1.178
19	431.477	4.5273	6	2	−2.929	90.023	−0.895

BBB, have good intestinal absorption and solubility. Overall the ADMET analysis and Lipinski's rule of 5 showed that these compounds are drug-like compounds (Table 2).

3. Conclusion

In conclusion we have synthesized nineteen benzimidazole analogues (**1–19**) and screened against urease inhibitory potential. All analogues showed a varied degree of urease inhibition with IC$_{50}$ values ranging between 0.90 ± 0.01 to 35.20 ± 1.10 μM when compared with the standard drug thiourea having IC$_{50}$ value 21.40 ± 0.21 μM. SAR studies were carried out to investigate the role of substitutions and nature of the functional groups attached to the phenyl ring which exert imperative influence on the urease inhibitory potential. Molecular docking study was performed to understand the binding interaction of the most active analogues with enzyme active site.

4. Material and methods

4.1. General procedure for the synthesis of benzimidazole analogues (1–19)

We mixed 1*H*-benzimidazole-2-thiol (**I**) (1 mmol) with methyl 4-(bromomethyl) benzoate (**II**) (1 mmol) in acetone in the presence of K$_2$CO$_3$ and reflux for 5 hrs to give methyl 4-(((1*H*-benzimidazol-2-yl)thio)methyl)benzoate (**III**) as intermediate product. The intermediate product (**III**) was further treated with hydrazine hydrate in ethanol and reflux for 6 hrs to give 4-(((1*H*-benzimidazol-2-yl)thio)methyl)benzohydrazide (**IV**) as second intermediate product. The intermediate product (**IV**) was finally treated and refluxed with different substituted aldehyde/acetophenone to give the desired products (**1–19**). Completion of reaction was monitored by TLC. After completion of reaction; the product was filtered, washed with *n*-hexane and dried.

4.1.1. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(2-nitrobenzylidene) benzohydrazide (1)

Yield: 85%. ^{1}H NMR (500 MHz, DMSO-*d$_6$*): δ 12.6 (s, 1H, NH), 12.1 (s, 1H, NH), 8.8 (s,1H, −N=CH), 8.16 (d, *J* = 6.5 Hz, 2H, Aromatic-H), 8.08 (d, *J* = 6.2 Hz, 1H, Aromatic-H), 7.89 (m, 3H, Aromatic-H), 7.81 (br. s, 1H, Aromatic-H), 7.72 (br. s, 1H, Aromatic-H), 7.67 (d, *J* = 6.5 Hz, 3H, Aromatic-H), 7.14 (s, 1H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-*d$_6$*): δ 163.7, 149.3, 147.4, 143.1, 140.0, 138.5, 134.4, 131.6, 131.2, 129.9 128.1, 127.5, 127.3, 127.1, 127.0, 123.9, 123.3, 123.1, 115.3, 115.1, 34.2 HREI-MS: *m/z* calcd for C$_{22}$H$_{17}$N$_5$O$_3$S [M]$^+$ 431.1052, Found 431.1049.

4.1.2. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(4-chlorobenzylidene) benzohydrazide (2)

Yield: 78%. ^{1}H NMR (500 MHz, DMSO-*d$_6$*): δ 12.6 (s, 1H, NH), 11.8 (s, 1H, NH), 8.7 (s,1H, −N=CH), 7.8 (d, *J* = 6.5 Hz, 2H, Aromatic-H), 7.7 (d, *J* = 6.7 Hz, 2H, Aromatic-H), 7.6 (m, 4H, Aromatic-H), 7.5 (t, *J* = 6.8 Hz, 1H, Aromatic-H), 7.3 (d, *J* = 7.2 Hz, 2H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-*d$_6$*): δ 163.5, 149.4, 146.5, 140.2, 138.6, 138.3, 136.6, 131.3, 131.0, 130.2, 128.5, 128.3, 127.4, 127.1, 126.9, 126.7, 123.4, 123.1, 115.2, 115.0, 34.1. HREI-MS: *m/z* calcd for C$_{22}$H$_{17}$ClN$_4$OS [M]$^+$ 420.0812, Found 420.0809.

4.1.3. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(3-nitrobenzylidene) benzohydrazide (3)

Yield: 85%. ^{1}H NMR (500 MHz, DMSO-*d$_6$*): δ 12.5 (s, 1H, NH), 12.1 (s, 1H, NH), 9.3 (s,1H, −N=CH), 8.3 (s, 1H, Aromatic-H), 8.1 (dd, *J* = 1.3, 6.5 Hz, 1H, Aromatic-H), 7.9 (dd, *J* = 1.2, 6.7 Hz, 1H, Aromatic-H), 7.8 (d, *J* = 6.6 Hz, 2H, Aromatic-H), 7.7 (t, *J* = 6.8 Hz, 1H, Aromatic-H), 7.6 (m, 2H, Aromatic-H), 7.5 (d, *J* = 6.3 Hz, 2H, Aromatic-H), 7.2 (m, 2H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-*d$_6$*): δ 163.4, 149.5, 147.8, 146.4, 140.1, 138.5, 138.3, 134.2, 132.2, 130.8, 129.4, 127.5, 127.2, 127.0, 126.7, 126.2, 123.4, 123.1, 115.1, 115.0, 34.2, 121.2. HREI-MS: *m/z* calcd for C$_{22}$H$_{17}$N$_5$O$_3$S [M]$^+$ 431.1052, Found 431.1049.

4.1.4. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(2,4-dichlorobenzylidene) benzohydrazide (4)

Yield: 65%. ^{1}H NMR (500 MHz, DMSO-*d$_6$*): δ 12.6 (s, 1H, NH), 12.4 (s, 1H, NH), 9.3 (s,1H, −N=CH), 8.3 (d, *J* = 6.1 Hz, 1H, Aromatic-H), 7.8 (d, *J* = 6.6 Hz, 2H, Aromatic-H), 7.7 (s, 1H, Aromatic-H), 7.5 (m, 2H, Aromatic-H), 7.4 (d, *J* = 7 Hz, 2H, Aromatic-H), 7.3 (d, *J* = 6.3 Hz, 1H, Aromatic-H), 7.2 (m, 2H, Aromatic-H 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-*d$_6$*): δ 162.6, 149.3, 140.2, 138.6, 138.4, 138.0, 132.3, 131.2, 131.0, 129.2, 129.0, 127.2, 127.1, 126.9, 126.5, 126.0, 126.0 123.3, 123.2, 115.3, 115.1, 34.5. HREI-MS: *m/z* calcd for C$_{22}$H$_{16}$Cl$_2$N$_4$OS [M]$^+$ 454.0422, Found 454.0418.

4.1.5. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(5-bromo-2-methoxybenzylidene) benzohydrazide (5)

Yield: 82%. ^{1}H NMR (500 MHz, DMSO-*d$_6$*): δ 12.4 (s, 1H, NH), 11.5 (s, 1H, NH), 9.3 (s,1H, −N=CH), 7.8 (d, *J* = 6.7 Hz, 2H, Aromatic-H), 7.7 (d, *J* = 1.2 Hz, 1H, Aromatic-H), 7.5 (m, 2H, Aromatic-H), 7.4 (d, *J* = 6.8 Hz, 1H, Aromatic-H) 7.3 (d, *J* = 6.8 Hz, 2H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 6.9 (d, *J* = 7.1 Hz, 1H, Aromatic-H), 4.6 (s, 2H,

−S−CH$_2$), 3.8 (s, 3H, −OCH$_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.3, 156.1, 149.4, 145.8, 139.9, 138.6, 138.2, 134.5, 131.1, 130.8, 127.4, 127.2, 126.9, 126.6, 123.4, 123.2, 118.7, 115.4, 115.3, 112.8, 109.8, 55.4, 34.5. HREI-MS: *m/z* calcd for C$_{23}$H$_{19}$BrN$_4$O$_2$S [M]$^+$ 494.0412, Found 494.0410.

4.1.6. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(3-hydroxy-4-methoxybenzylidene) benzohydrazide (6)

Yield: 82%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (s, 1H, NH), 11.4 (s, 1H, NH), 9.3 (s,1H, −N=CH), 7.8 (d, *J* = 6.1 Hz, 2H, Aromatic-H), 7.6 (m, 2H, Aromatic-H), 7.5 (m, 3H, Aromatic-H), 7.4 (s, 1H, Aromatic-H), 7.2 (m, 2H, Aromatic-H), 6.9 (d, *J* = 7.3 Hz, 1H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$), 3.8 (s, 3H, −OCH$_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.4, 151.7, 149.5, 146.6, 146.3, 139.8, 139.2, 138.9, 131.2, 131.0, 127.6, 127.2, 127.0, 127.0, 123.4, 123.2, 122.5, 115.5, 115.1, 114.9, 111.8, 34.2, 55.9. HREI-MS: *m/z* calcd for C$_{23}$H$_{20}$N$_4$O$_3$S [M]$^+$ 432.1256, Found 432.1252.

4.1.7. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(4-(benzyloxy) benzylidene) benzohydrazide (7)

Yield: 75%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (s, 1H, NH), 11.6 (s, 1H, NH), 8.3 (s,1H, −N=CH), 7.8 (d, *J* = 6.8 Hz, 2H, Aromatic-H), 7.6 (d, *J* = 7.2 Hz, 2H, Aromatic-H), 7.5 (d, *J* = 6.7 Hz, 2H, Aromatic-H), 7.4 (m, 4H, Aromatic-H), 7.3 (t, *J* = 7.8 Hz, 2H, Aromatic-H), 7.1 (m, 5H, Aromatic-H), 5.1 (s, 2H, −OCH$_2$) 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.6, 159.8, 1493, 146.5, 140.6, 138.7, 138.7, 132.4, 132.4, 130.2, 130.2, 129.8, 128.8, 128.8, 127.5, 127.3, 127.1, 126.9, 126.7, 126.4, 126.0, 123.4, 123.2, 115.1, 115.1, 114.4, 114.4, 69.4, 34.2. HREI-MS: *m/z* calcd for C$_{29}$H$_{24}$N$_4$O$_2$S [M]$^+$ 492.1620, Found 492.1617.

4.1.8. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(1-(4-nitrophenyl) ethylidene) benzohydrazide (8)

Yield: 85%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.4 (s, 1H, NH), 11.6 (s, 1H, NH), 8.3 (d, *J* = 7.6 Hz, 2H, Aromatic-H), 8.1 (d, *J* = 7.3 Hz, 2H, Aromatic-H), 7.8 (d, *J* = 6.9 Hz, 2H, Aromatic-H), 7.7 (dd, *J* = 1.2, 6.7 Hz, 2H, Aromatic-H), 7.4 (d, *J* = 6.5 Hz, 2H, Aromatic-H), 7.2 (m, 2H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$), 2.6 (s, 3H, −N=CH$_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.4, 150.0, 149.3, 147.2, 143.2, 140.1, 138.5, 138.4, 130.8, 126.7, 127.5, 127.3, 127.1, 127.0, 126.6, 126.5, 126.2 123.2, 122.9, 115.1, 114.9, 34.4, 16.3. HREI-MS: *m/z* calcd for C$_{23}$H$_{19}$N$_5$O3S [M]$^+$ 445.1209, Found 445.1205.

4.1.9. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(3,4-dichlorobenzylidene) benzohydrazide (9)

Yield: 78%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (s, 1H, NH), 12.01 (s, 1H, NH), 8.3 (s,1H, −N=CH), 7.9 (s, 1H, Aromatic-H), 7.8 (d, *J* = 6.4 Hz, 2H, Aromatic-H), 7.7 (s-br, 2H, Aromatic-H), 7.6 (d, *J* = 6.6 Hz, 2H, Aromatic-H), 7.3 (d, *J* = 6.9 Hz, 2H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.4, 149.5, 146.4, 140.1, 138.5, 138.4, 135.2, 133.2, 133.0, 131.0, 130.3, 130.2, 128.5, 127.4, 127.3, 127.1, 127.0, 122.9, 122.7, 114.8, 114.6, 34.3. HREI-MS: *m/z* calcd for C$_{22}$H$_{16}$Cl$_2$N$_4$OS [M]$^+$ 454.0422, Found 454.0418.

4.1.10. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(1-(2-hydroxyphenyl) ethylidene) benzohydrazide (10)

Yield: 68%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.5 (s, 1H, NH), 11.2 (s, 1H, NH), 7.87 (d, *J* = 7.3 Hz, 2H, Aromatic-H), 7.82 (t, *J* = 7.2 Hz, 1H, Aromatic-H), 7.5 (dd, *J* = 1.3, 7.3 Hz, 2H, Aromatic-H), 7.4 (d, *J* = 6.9 Hz, 2H, Aromatic-H), 7.3 (t, *J* = 6.5 Hz, 1H, Aromatic-H), 7.2 (m, 4H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$), 2.6 (s, 3H, −N=CH$_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.3, 163.4, 162.1, 149.4, 140.2, 138.5, 138.3, 132.1, 131.7, 131.5, 127.4, 127.3, 126.8, 126.5, 123.1, 123.0, 121.1, 118.5, 117.5, 115.1, 115.0, 34.2, 16.8. HREI-MS: *m/z* calcd for C$_{23}$H$_{20}$N$_4$O$_2$S [M]$^+$ 416.1307, Found 416.1304.

4.1.11. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(anthracen-9-ylmethylene) benzohydrazide (11)

Yield: 65%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.6 (s, 1H, NH), 12.07 (s, 1H, NH), 9.6 (s, 1H, −N=CH$_3$),8.77 (d, *j* = 7.2 2H, Aromatic-H), 8.72 (s, 1H, Aromatic-H), 8.1 (d, *j* = 6.5, 2H, Aromatic-H), 7.9 (d, *j* = 6.1, 2H, Aromatic-H), 7.69 (m, 4H, Aromatic-H), 7.60 ((d, *j* = 6, 3H, Aromatic-H), 7.4 (s-br, 1H, Aromatic-H), 7.1 (s-br, 2H, Aromatic-H), 4.7 (s, 2H, −SCH$_2$), [1]C NMR (125 MHz, DMSO, d_6): δ162.7, 149.3, 146.9, 143.5, 142.0, 138.8, 135.4, 132.3, 130.9, 130.8, 129.5, 129.3, 128.9, 128.2, 127.8, 127.1, 125.5, 125.0, 124.8, 121.7, 121.6, 121.4, 121.3, 121.2, 121.2, 117.5, 117.4, 117.3, 110.3, 34.3. HREI-MS: *m/z* calcd for C$_{30}$H$_{22}$N$_4$OS [M]$^+$ 486.1514, Found 486.1511.

4.1.12. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(1-(2-hydroxyphenyl) ethylidene) benzohydrazide (12)

Yield: 80%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (s, 1H, NH), 12.07 (s, 1H, NH), 7.8 (d, *J* = 6 Hz, 2H, Aromatic-H), 7.6 (m, 3H, Aromatic-H) 7.5 (m, 3H, Aromatic-H), 7.3 (m, 2H, Aromatic-H), 7.1 (dd, *J* = 1.6, 6.4 Hz, 1H, Aromatic-H), 6.8 (dd, *J* = 1.9, 7.4 Hz, 1H, Aromatic-H), 4.6 (s, 2H, −SCH$_2$), 2.6 (s, 3H, −N=CH$_{33}$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.4, 160.9, 149.3, 147.3, 140.2, 138.4, 138.3, 136.2, 130.9, 129.2, 128.5, 128.3, 128.1 128.2, 127.4, 127.3, 127.2, 126.8, 126.7, 126.4, 126.3, 123.3, 123.2, 115.2, 115.1, 113.9, 113.8, 70.4, 34.3, 16.4. HREI-MS: *m/z* calcd for C$_{23}$H$_{20}$N$_4$O$_2$S [M]$^+$ 416.1307, Found 416.1304.

4.1.13. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(4-(dimethylamino) benzylidene) benzohydrazide (13)

Yield: 82%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (s, 1H, NH), 11.4 (s, 1H, NH), 8.2 (s, 1H, −N=CH$_3$), 7.8 (d, *J* = 6.7 Hz, 2H, Aromatic-H), 7.5 (m, 6H, Aromatic-H), 7.1 (m, 2H, Aromatic-H) 6.7 (d, *J* = 7.3 Hz, 2H, Aromatic-H), 4.6 (s, 2H, −SCH$_2$), 2.9 (s, 6H, −N−(CH$_3$)$_2$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.6, 152.8, 149.3, 146.3, 140.2, 138.3, 138.1, 130.8, 128.3, 128.1, 127.6, 127.3, 127.2, 127.1, 123.4, 123.2, 122.9, 115.3, 115.2, 111.5, 111.3, 40. 8, 40.8, 34.4. HREI-MS: *m/z* calcd for C$_{24}$H$_{23}$N$_5$OS [M]$^+$ 429.1623, Found 429.1620.

4.1.14. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(4-hydroxy-3-methoxybenzylidene) benzohydrazide (14)

Yield: 75%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (s, 1H, NH), 11.1 (s, 1H, NH), 9.3 2 (s, 1H, −N=CH$_3$), 7.8 (d, *J* = 6.3 Hz, 2H, Aromatic-H), 7.6 (m, 3H, Aromatic-H), 7.5 (m, 2H, Aromatic-H), 7.3 (s-br, 1H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 6.3 (d, *J* = 6.7, 1H, Aromatic-H), 4.6 (s, 2H, −SCH$_2$), 3.8 (s, 3H, −OCH$_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.4, 150.8, 149.6, 149.4, 146.5, 140.2, 138.4, 138.3, 130.8, 130.5, 127.4, 127.2, 127.1, 127.0, 123.2, 123.0, 122.6, 116.8, 115.4, 115.2, 111.7, 55.9, 34.2. HREI-MS: *m/z* calcd for C$_{23}$H$_{20}$N$_4$O$_3$S [M]$^+$ 432.1256, Found 432.1252.

4.1.15. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(1-(2,4-dihydroxyphenyl) ethylidene) benzohydrazide (15)

Yield: 60%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.5 (s, 1H, NH), 11.6 (s, 1H, NH), 7.8 (d, *J* = 6.3 Hz, 2H, Aromatic-H), 7.5 (m, 3H, Aromatic-H), 7.4 (m, 3H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 6.8 (d, *J* = 6.2 Hz, 1H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$), 2.7 (s, 3H, −N=CH$_{33}$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.5, 163.3, 162.2, 162.1, 149.3, 140.1, 138.5, 138.3, 130.9, 129.5, 127.5, 127.2, 127.1, 127.0, 123.3, 123.1, 115.3, 115.1, 111.2, 108.1, 103.2, 34.3, 16.7. HREI-MS: *m/z* calcd for C$_{23}$H$_{20}$N$_4$O$_3$S [M]$^+$ 432.1256, Found 432.1252.

4.1.16. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(4-hydroxybenzylidene) benzohydrazide (16)

Yield: 63%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (s, 1H, NH), 11.6 (s, 1H, NH), 9.9 (s, 1H, −N=CH$_3$), 7.8 (d, *J* = 6.6 Hz, 2H, Aromatic-H), 7.6 (d, *J* = 6.2 Hz, 2H, Aromatic-H), 7.5 (dd, *J* = 1.4, 6.8 Hz, 2H,

Aromatic-H), 7.4 (d, *J* = 6.2 Hz, 2H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 6.9 (d, *J* = 6.2 Hz, 2H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.4, 160.4, 149.4, 146.3, 140.1, 138.3, 138.2, 131.1, 130.2, 130.2, 127.3, 127.2, 127.1, 127.1, 125.8, 123.2, 123.1, 116.0, 116.0, 115.2, 115.1, 34.3. HREI-MS: *m/z* calcd for C$_{22}$H$_{18}$N$_4$O$_2$S [M]$^+$ 402.1150, Found 402.1146.

4.1.17. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(3,5-dichloro-2-hydroxybenzylidene) benzohydrazide (17)

Yield: 70%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (s, 1H, NH), 11.3 (s, 1H, NH), 8.5 (s, 1H, −N=CH$_3$), 7.9 (d, *J* = 7.6 Hz, 2H, Aromatic-H), 7.7 (m, 2H, Aromatic-H), 7.5 (s,1H, Aromatic-H), 7.4 (d, *J* = 8.1 Hz, 2H, Aromatic-H), 7.3 (s,1H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.5, 157.5, 149.2, 145.7, 139.9, 138.5, 138.3, 133.8, 130.7, 128.2, 128.1, 127.3, 127.2, 127.1, 127.1, 126.4, 123.3, 123.1, 122.8, 115.3, 115.2, 34.3. HREI-MS: *m/z* calcd for C$_{22}$H$_{16}$Cl$_2$N$_4$O$_2$S [M]$^+$ 470.0371, Found 470.0367.

4.1.18. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(3,4-dihydroxybenzylidene) benzohydrazide (18)

Yield: 74%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.4 (s, 1H, NH), 11.6 (s, 1H, NH), 8.2 (s, 1H, −N=CH$_3$), 7.8 (d, *J* = 6.1 Hz, 2H, Aromatic-H), 7.7 (m, 2H, Aromatic-H), 7.6 (d, *J* = 6.4 Hz, 2H, Aromatic-H), 7.4 (s, 1H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 6.9 (d, *J* = 6.3 Hz, 1H, Aromatic-H), 6.8 (d, *J* = 6.2 Hz, 1H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.5, 149.3, 149.1, 146.4, 145.9, 140.3, 138.4, 138.3, 131.1, 131.0, 127.4, 127.3, 127.2, 127.2, 123.4, 123.2, 123.0, 117.1, 115.9, 115.3, 115.2, 34.3. HREI-MS: *m/z* calcd for C$_{22}$H$_{18}$N$_4$O$_3$S [M]$^+$ 418.1100, Found 418.1097.

4.1.19. 4-((1H-benzimidazol-2-yl-thio)methyl)-N′-(4-nitrobenzylidene) benzohydrazide (19)

Yield: 83%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.7 (s, 1H, NH), 11.2 (s, 1H, NH), 8.7 (s, 1H, −N=CH$_3$), 8.2 (d, *J* = 7.4 Hz, 2H, Aromatic-H), 7.9 (d, *J* = 6.4 Hz, 2H, Aromatic-H), 7.8 (d, *J* = 8.2 Hz, 2H, Aromatic-H), 7.7 (m, 2H, Aromatic-H), 7.6 (d, *J* = 6.9 Hz, 2H, Aromatic-H), 7.1 (m, 2H, Aromatic-H), 4.6 (s, 2H, −S−CH$_2$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.4, 150.1, 149.2, 146.3, 140.2, 139.3, 138.4, 138.3, 130.7, 127.4, 127.3, 127.1, 127.1, 124.4, 124.3, 124.1, 124.1, 123.3, 123.2, 115.3, 115.2, 34.2. HREI-MS: *m/z* calcd for C$_{22}$H$_{17}$N$_5$O$_3$S [M]$^+$ 431.1052, Found 431.1049.

4.2. Urease assay protocol

The reaction mixtures, comprising 25 μL of enzyme solution and 55 μL of buffers containing 100 mM urea, were incubated with 5 μL of the test compounds (0.5 mM concentration) at 30 °C for 15 min in 96-well plates. For the kinetics assessment the urea concentrations were changed from 2 to 24 mM. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn [36]. Briefly, 45 μL of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and, 70 μL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 μL. The results (change in absorbance per min) were processed by using SoftMaxPro software (molecular Device, USA). The entire assays were performed at pH 6.8. Percentage inhibition was calculated from the formula 100-(OD$_{test\ well}$/OD$_{control}$) × 100. Thiourea was used as the standard inhibitor for urease.

Acknowledgment

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