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1,3,5-Thiadiazinane thione derivatives as significant urease inhibitors

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Abstract: We report the promising urease inhibitory activity of four sets of tetrahydro thiadiazine thiones (THTT) namely 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine thiones: THTT 5-8 (set A) having alkyl/aryl substituents at *N*-3 and *N*-5 positions; THTT 9-12 (set B) and THTT 13-14 (set C) with 3-carboxylic acid derivatives and tetrahydro-2*H*-1,3,5-thiadiazine-6-thione esters 15-16 (set D). Gratifyingly, all four sets of THTT were recognized as promising inhibitors of urease enzyme. Among 12 tested compounds; THTT 6, 8, 10, 14 and 15 from each set respectively, demonstrated significant urease inhibitory activity with IC_{50} values between 11.2-29.8 μM which is mostly found higher than that for thiourea, a standard urease inhibitor with IC_{50} value of 22.4 μM. Furthermore, compound 7 showed almost the same level of inhibition (IC_{50} = 22.5 μM) as of standard. In addition, molecular docking study supported the phenomenon that thiadiazinane ring itself is an active pharmacophore that binds through CH_2 groups and S atom *via* carbon-hydrogen/π-sulfur interactions respectively to the active site of the urease enzyme. The optimistic results from this study suggest the use of thiadiazinane skeleton as a guided template for the advancement of new urease inhibitors in drug discovery.

Keywords: Tetrahydro-1,3,5-thiadiazine thiones, urease inhibition, thiourea, molecular docking, drug discovery.

INTRODUCTION

Urease enzyme facilitates the survival of microorganisms in humans and thus contributes in many types of infections, for example, gastric ulcers and cancers caused by helicobacter pylori which propagate in the acidic environment of stomach (Hameed *et al*., 2019). This enzyme also causes urolithiasis (stone formation in kidney), pyelonephritis (inflammation of the kidney caused by bacterial infection), hepatic encephalopathy (weakening in brain function as a result of liver disease), hepatic coma (sum of neuropsychiatric disorders) and urinary catheter encrustation (Mobley *et al*., 1995; Mobley and Hausinger, 1989). Existing medications for the treatment of urease-related infections are mostly ineffective (Bayerdörffer and Ottenjann, 1988); hence there is an immense need of substitute treatment. In this context, the investigation of effective urease inhibitors got the interest of researchers worldwide (Kafarski and Talma, 2018). And in recent times, different types of compounds have been reported as urease inhibitors including hydroxamic acid derivatives (Kobashi *et al*., 1975), benzimidazoles (Kuehler *et al*., 1995), barbiturates/thiobarbiturates (Rauf *et al*., 2011; Qureshi *et al*., 2015), and dihydropyrimidines (Khan *et al*., 2016).

Thus, there is an alarming absence of urease inhibitor drugs available to the market. Unfortunately, acetohydroxamic acid (Lithostat) is the only available urease inhibitor drug till now which is also known for many side effects as well (Hameed *et al*., 2019). Therefore, there is immense need to discover new and effective urease inhibitors. Among bioactive heterocycles (fig. 1), tetrahydro-2*H*-1,3,5-thiadiazine thione (THTT) derivatives received considerable attention of researchers because of their diverse biological activities that include antiprotozoal (compound 1) (Coro *et al*., 2008), antitubercular (compound 2) (Katiyar *et al*., 2003), leishmanicidal (compound 3) (Monzote *et al*., 2004), anticancer (Radwan *et al*., 2012) and antifungal activities (Saglam *et al*., 2011).

Herein, we report the investigation of various derivatives of thiadiazinane (5-16) in four different sets (as shown in scheme 1) for their comparative urease inhibitory activity against jack bean urease along with the molecular docking studies of the least active, most active and ester derivate of most active analogue.

MATERIALS AND METHODS

General experimental details

NMR spectra of compounds were acquired on Bruker AV-400 and Bruker AV-500 instruments. Mass spectrometry was performed using ESI (electrospray ionization) and EI (Electron ionization) techniques on

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QSTAR XL MS/MS (Applied Biosystems) and JMS-600H (JEOL), respectively.

The synthesized THTT products (5-16 of sets A, B, C and D) were purified *via* recrystallization or column chromatography. Chemical reagents were obtained commercially from Sigma Aldrich.

Synthetic Methods and Spectroscopic Data

All the THTT analogues (5-16 of sets A, B, C and D) have been prepared as shown in scheme 1 for their biological evaluation following the literature protocol (Arshad *et al*., 2018).

General procedure for the synthesis of THTT analogues (5-8 of set A)

To a stirred solution of 10 mmol phenylethylamine, or alkyl amine (4) in 10 mL absolute ethyl alcohol containing 10 mmol KOH; 60 mmol of CS_2 was introduced and stirring continued for 3 hours duration at 25 °C. Afterwards, with gradual inclusion of 35% formaline (22 mmol) the reaction mixture was stirred for 1 hour. Thereafter, following clear mixture was added gradually to a solution of 10 mmol alkyl amine in phosphate buffer (20 mL of 7.8 pH). After subsequent 1 hour stirring, mixture was cooled upto 0 °C and acidification (pH 2) was performed with hydrochloric acid (8%). Furthermore, after half an hour stirring, precipitates were filtered and recrystallized by ethyl alcohol to provide THTT 5-8 (set A) in pure form. The spectral data of THTT compounds were found well matched with the reported data (Arshad *et al*., 2018).

General procedure for the synthesis of THTT analogues (9-12 of set B)

THTT analogues (9-12) were prepared following the above mentioned methodology of set A except that in the last stage, instead of alkyl amines, 10 mmol of glycine was used. The spectral data of isolated THTT compounds were found well matched with the reported data (Aboul-Fadl and El-Shorbagi, 1996; Arshad *et al*., 2018).

General procedure for the synthesis of THTT analogues (13-14 of set C)

THTT analogues (13-14) were prepared following the earlier mentioned methodology of set A except that in the first stage, instead of phenylethylamine or alkyl amines, 10 mmol of glycine was used. The spectral data of isolated THTT compounds were found well matched with the reported data (Hussein and Hashem, 2008; Arshad *et al*., 2018).

General procedure for the synthesis of THTT ester analogues (15-16, set D)

THTT analogues (15-16) were prepared by taking 1 mmol of *N*-3 carboxylic group derivative of THTT 10 and 12 (set B) in 1.5 mL of ethyl alcohol at ice cooled environment. Thereafter, 1.5 mmol of thionyl chloride was introduced dropwise to the above stirred mixture approximately in 10 minutes and stirred for further 2 hours before evaporating ethyl alcohol. Then water (25 mL) was added to the mixture and extraction was performed 3 times from ethyl acetate *via* separating funnel. Combined ethyl acetate solution was washed with saturated $NaHCO_3$ (10 mL) then ethyl acetate was dried over $MgSO_4$ and filtered. Evaporation of solution delivered crude THTT ester which was subsequently purified *via* column chromatography to provide THTT 15-16 (set D) in pure form. The spectral data of isolated THTT compounds were found well matched with the reported data (Ullah *et al*., 2021; Arshad *et al*., 2018).

Materials for biological assay protocol

The Jack bean urease was obtained from Biowest, France having Cat No. S181H-100 and Lot No. S11302S181H.

Urease inhibition assay

The antiurease activities of THTT compounds (5-16, >95% NMR pure) were experimented in 96-well microplates (flat bottomed) by following the standardized assay protocol (Khan *et al*., 2016). Microplates were loaded with jack bean urease enzyme (25 µL), buffer (55 µL with 100 mM urea) and 1 mM THTT analogues (5 µL). After 15 minutes incubation at 30 °C, urease activity was determined on the basis of ammonia produced and adopting the Weatherburn indophenol method (Weatherburn, 1967). For this objective, phenol reagent (1% phenol + 0.005% sodium nitroprusside by w/v) 45 µL and alkaline reagent (0.5% NaOH and 0.1% NaOCl by w/v) 70 µL were included in each micro-well. The absorbance was measured by microplate reader device equipped with SoftMax Pro software (USA) at 630 nm. All the testings were examined thrice and for a total volume of 200 µL. Buffer of pH 8.2 containing 1 mM EDTA, 0.01 M $LiCl_2$, 0.01 M K_2HPO_4 was used. Thiourea was utilized as standard urease inhibitory agent.

Experimental Details of Molecular Docking

Jack bean urease enzyme (PDBID 4H9M) structure was fetched from the PDB (Protein Data Bank) (Benini *et al*., 2000). Crystal conformation of the enzyme in stable state was saved by Chimera 1.10.1 tool (The Univ. of California, USA). The stereochemistry of the urease, Prot Param and Ramachandran plot values were calculated by the server link: http://molprobity.biochem.duke.edu. Discovery study client tool 4.1 (BIOVIA 5005 Wateridge Vista Drive, USA) was utilized to achieve hydrophobicity plots of targeted protein. In addition, beta sheets, turns, coils, percent statistics of receptor protein helices and protein architectures were predicted by VANDAR 1.8 (online server) (Rashid *et al*., 2020).

The THTT analogue 5 (least active), 10 (most active) and 15 (ester derivative of THTT 10) stable structures were fetched using Chimera 1.10.1 tool and the structures were sketched through ACD/ChemSketch. Target protein active sites were evaluated by protein data bank (PDB) then molecular docking experiments were carried out by

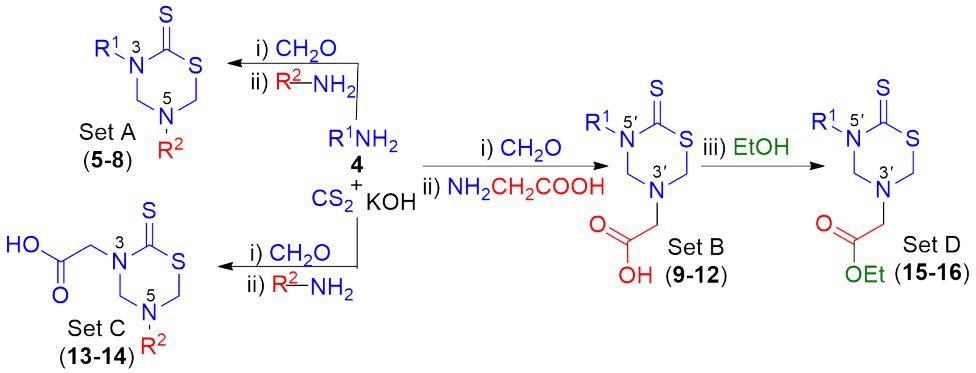
Table 1: Urease inhibitory activities of THTT compounds 5-16 (sets A–D).[a]

S. No.	Compound	R^1	R^2	Urease Inhibition IC_{50} ± SEM[b] (µM)
Set A				
1.	5	–$CH(CH_3)Ph$	–C_3H_7	67.9 ± 0.51
2.	6	–$CH(CH_3)Ph$	–*i*-Pr	18.7 ± 0.59
3.	7	–$CH(CH_3)Ph$	–C_2H_4OH	22.5 ± 0.11
4.	8	–C_2H_5	–C_2H_5	18.9 ± 0.83
Set B				
5.	9	–*i*-Pr	–CH_2COOH	34.5 ± 0.33
6.	10	–C_3H_7	–CH_2COOH	11.2 ± 0.73
7.	11	–CH_2Bz(3,4-di-MeO)	–CH_2COOH	28.9 ± 0.69
8.	12	–$CH(CH_3)Ph$	–CH_2COOH	31.2 ± 0.96
Set C				
9.	13	–CH_2COOH	–C_3H_7	32.6 ± 0.63
10.	14	–CH_2COOH	–C_2H_4OH	29.8 ± 0.38
Set D				
11.	15	–C_3H_7	–	17.0 ± 0.37
12.	16	–$CH(CH_3)Ph$	–	26.7 ± 0.37
Standard				
13.[c]	Thiourea	–	–	22.4 ± 0.47

[a] Urease inhibitory activities are shown as the mean of triplicate testings. [b] SEM = ± Standard error of the mean. [c] IC_{50} value of thiourea = 22.4 µM as standard for urease inhibitory activity.

Table 2: Interaction results with the protein 4H9M.

S No.	Compound	Attractive charges	Pi-anion	Pi-Pi stacked	C-H bond	Metal acceptor	Pi-Alkyl/ Alkyl	Un-favorable Positive-Positive	Conven-tional H-bond	Pi-S
1	Thiourea				Asn789 Ile686					
2	5	Asp730	Asp 730	Phe712	Val7444	Val744	Met746 Tyr32 Lys716 Val744 Val36	Lys716		
3	10				Ala636 His409		His593 His519		Gly550 His492	His 492
4	15						Ala47 Ala85 Arg48 Arg6 Lys10		Thr86	



Scheme 1: Synthetic procedure to tetrahydro thiadiazine thiones 5-16. Reagents and conditions: i) 1° amines (10 mmol), EtOH (10 mL), KOH (1 equiv.), CS_2 (6 equiv.), rt, 3 hours; then CH_2O (2.2 equiv.), rt, 1 hour; ii) 1° amines (1 equiv.), phosphate buffer of pH 7.8 (20 mL), rt, 1 hour; iii) THTT (1 mmol), $SOCl_2$ (1.5 equiv.), EtOH (1.5 mL), 0–4 °C, 2 hours.

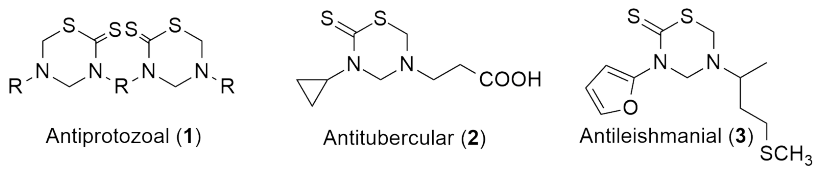


Fig. 1: Important bioactive molecules containing THTT skeleton.

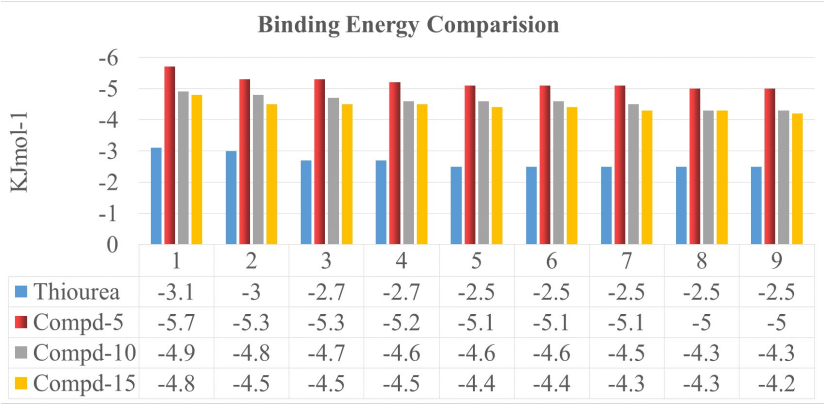


Fig. 2: Comparison of binding energies.

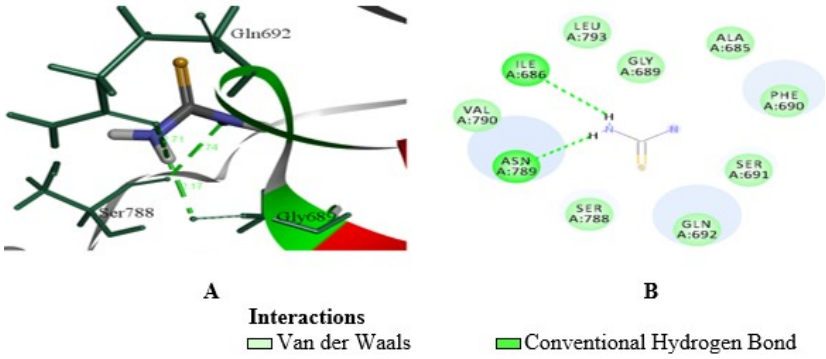


Fig. 3: (A) Represents 3D while (B) represents 2D binding site interactions of docked thiourea as standard.

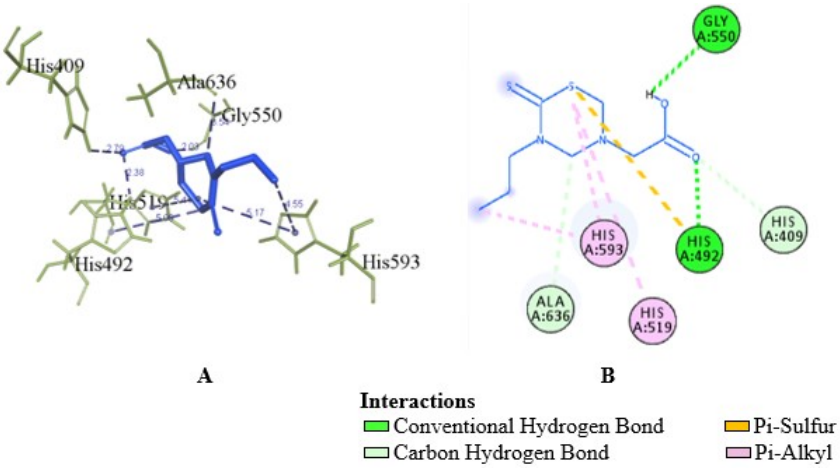


Fig. 4: (A) Represents 3D while (B) represents 2D binding site interactions of docked compound 10 (most active THTT)

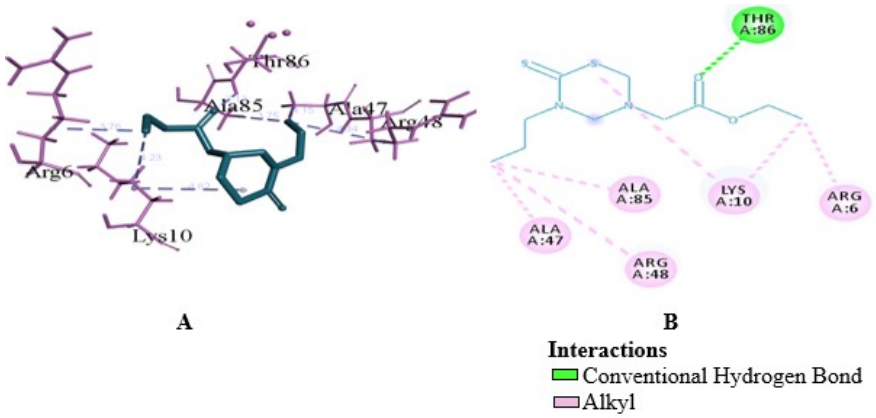


Fig. 5: (A) Represents 3D while (B) represents 2D binding site interactions of docked compound 15 (THTT ester)

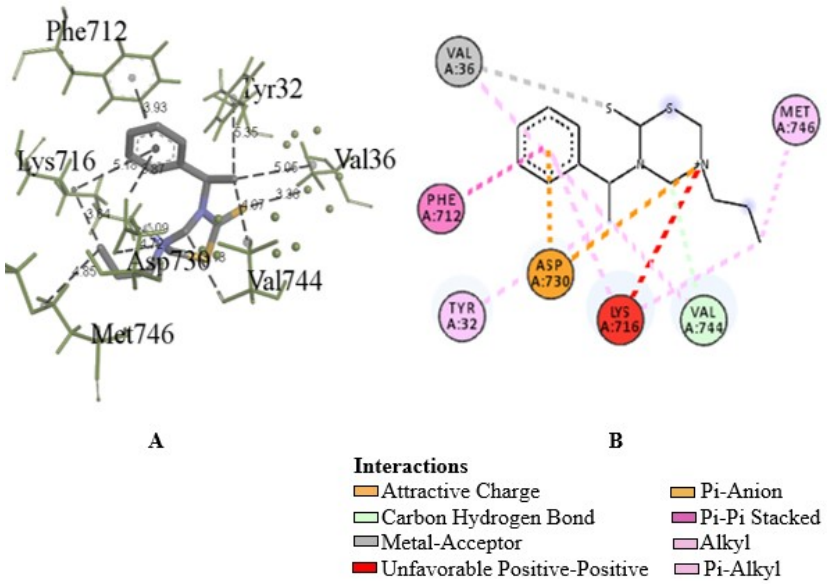


Fig. 6: (A) Represents 3D while (B) represents 2D binding site interactions of docked compound 5 (least active THTT)

CB-docking. Docked ligand-protein interactions/3-dimensional graphs were obtained from BIOVIA Discovery Studio Visualizer 2.1.0.

STATISTICAL ANALYSIS

All values in table 1 were expressed as standard error of mean (± SEM) and tests were performed in triplicate. Microplate reader from Molecular Devices, CA, USA was used for measuring the absorbance at 630 nm. The EZ-Fit Enzyme Kinetic Program (Perrella Scientific Inc., Massachusetts, USA) was employed to calculate the IC_{50} values.

RESULTS

3,5-Disubstituted tetrahydro-2*H*-1,3,5-thiadiazine thiones (THTT) 5-16 were prepared in four different sets, comprising 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thiones 5-8 (set A) having both (*N*-3 and *N*-5) alkyl/aryl substituents, disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-6-thiones 9-12 (set B), 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thiones 13-14 (set C) and disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-6-thione esters 15-16 (set D) as shown in scheme 1 in good to excellent yields (65-86%). The detailed synthesis of these compounds (sets A-D) and structural characterization was reported in literature (Ullah *et al*., 2021).

Urease inhibition

All four sets of thiadiazinane compounds (>95% NMR pure) having *N*-3 and *N*-5 alkyl/aryl substituents were examined for their *in vitro* urease inhibition potential against jack bean urease following standard protocol (Khan *et al*., 2016; Weatherburn, 1967). Gratifyingly, all four sets of THTT showed promising urease inhibitory activity and the results are drafted in table 1.

Molecular docking study

To examine the binding interaction of the THTT compounds, the molecular docking studies were also performed between the most active THTT analogue 10 of set B, its ester derivative 15 of set D, the least active compound 5 of set A and the urease enzyme (standard). The molecular docking experiment and observed binding energies (fig. 2) showed that the least active compound 5 had much difference in binding energies (-5.7 $kJmol^{-1}$ to -5.1 $kJmol^{-1}$) than that of standard thiourea (-3.1 $kJmol^{-1}$ to -2.6 $kJmol^{-1}$); however, the most active compound 10 and its ester derivative 15 had almost similar binding energy values i.e., (-5.0 $kJmol^{-1}$ to -4.7 $kJmol^{-1}$) and (-4.9 $kJmol^{-1}$ to -4.5 $kJmol^{-1}$) respectively, which are in complete agreement with the experimental urease inhibition potential.

DISCUSSION

Gratifyingly, all the compounds showed significant urease inhibitory potential against jack bean urease. THTT analogue 10 of set B with the *n*-propyl group attached at *N*-5′ and CH_2COOH fragment attached at *N*-3′ position was found the most potent compound (IC_{50} = 11.2 ± 0.73 µM, entry 6, table 1) among the tested compounds which was 2-fold more active than the thiourea used as standard urease inhibitor (IC_{50} = 22.4 ± 0.11 µM, entry 13, table 1). Similarly, its ethyl ester derivative 15 of set D was also found as the second most active compound (IC_{50} = 17.0 ± 0.37 µM, entry 11, table 1). These results showed the importance of *n*-propyl group at *N*-5′ position and CH_2COOH fragment at *N*-3′ position of the THTT skeleton. Furthermore, note that the alteration of these substituents as in compound 13 of set C, i.e., *n*-propyl group at *N*-5 position and CH_2COOH fragment at *N*-3 position resulted in the decrease of activity (32.6 ± 0.63 µM, entry 9, table 1). The THTT analogues 6 and 8 of set A were also found more active than the standard having IC_{50} values of 18.7 ± 0.59 µM and 18.9 ± 0.83 µM, respectively as shown in entries 2 and 4 of table 1. In addition to earlier mentioned, all other compounds of sets A–D except compounds 5 (set A), showed notable activities between the range of IC_{50} = 22.5 to 34.5 µM that is very much comparable to the standard urease inhibitor (thiourea).

From the molecular docking experiments as shown in fig. 4, it is observed that most active compound 10 showed interactions with six amino acid residues namely His519, His492, Gly550, Ala636, His593 and His409; adopting suitable orientation to the active site pocket of the urease enzyme. These interactions include two conventional hydrogen bonds, one carbon-hydrogen interaction, one pi-sulfur and three pi-alkyl interactions. It is important to note that compound 10 showed two conventional hydrogen bond interactions from COOH group at *N*-5′ position of the THTT skeleton with His492 and Gly550. However, only one conventional hydrogen bond interaction was observed in its ester analogue 15 (fig. 5) while the experimentally least active compound 5 lacks this interaction (fig. 6). Similarly compound 10 interacted through pi-sulfur interaction with His492 of the urease which was not observed in both other compounds 5 and 15. Therefore, the deficiency of these interactions in compound 5 and 15 might be the reason of lower urease inhibitory activity. This study also indicated the importance of CH_2COOH fragment at *N*-3′ position of the THTT nucleus. Additionally, one carbon hydrogen interaction was also observed through CH_2 group present at position-4 of THTT nucleus in compounds 5 and 10. It showed that THTT nucleus itself has significant interaction with active site pocket of urease enzyme. The interaction results with protein 4H9M are also summarized in table 2.

CONCLUSION

In conclusion, we report the promising urease inhibitory activity of four sets of tetrahydro thiadiazine thiones. All four sets of compounds were recognized as significant inhibitors of urease enzyme. Among them, THTT analogue 10 showed potent activity (IC_{50} = 11.2 µM) which is 2-fold higher than the standard urease inhibitor thiourea (IC_{50} = 22.4 µM). Furthermore, compounds 15, 6 and 8 also exhibited excellent urease inhibitory activity (IC_{50} = 17.0, 18.7 and 18.9 µM, respectively) when compared with thiourea as standard urease inhibitor. In addition, except compound 5, all THTT compounds showed activity between the range of IC_{50} = 22.5 to 34.5 µM). The *in vitro* activity results were also supported by the molecular docking study of the most potent THTT analogue 10 along with its ester analogue 15 and least active THTT analogue 5. The study indicated that the thiadiazinane ring itself is an active pharmacophore that binds through its various atoms *via* carbon hydrogen and π-sulfur interactions to the active binding pocket of the urease enzyme. Furthermore, strong interactions were found in most active THTT analogue 10 other than the compounds 5 and 15. The optimistic results from this study suggest the use of thiadizinane skeleton as a guided template for the development of new urease inhibitory drugs.

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