2-Nitrobenzohydrazide as a Potent Urease Inhibitor: Synthesis, Characterization and Single Crystal X-ray Diffraction Analysis

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(Received on 1st August 2016, accepted in revised form 3rd July 2017)

Summary: 2-Nitrobenzohydrazide was efficiently synthesized in two steps by the esterification of 2-nitrobenzoic acid followed by the treatment with hydrazine hydrate in methanol. The structure of 2-nitrobenzohydrazide was established by modern spectro-analytical techniques including FTIR, ^{1}H and ^{13}C-NMR spectroscopy and unequivocally confirmed by single crystal X-ray diffraction data. 2-Nitrobenzohydrazide crystallized in orthorhombic space group P 21 21 21 with unit cell dimensions a = 4.9764(4) Å, b = 12.5280 (3) Å, c = 12.8512(1) Å, $\alpha = \beta = \gamma = 90°$. A combination of N–H…N and N–H…O hydrogen bonds stabilized the crystal packing of **3**. The synthesized compound **3** was assessed as urease inhibitor against *Jack bean* urease and the results revealed good inhibitory potency with an IC_{50} value of 4.25 ± 0.08 μM. This inhibition strength was 5-fold higher compared to the reference inhibitor thiourea (IC_{50} = 21.00 ± 0.11 μM). The molecular docking studies of the synthesized inhibitor **3** inside the active pocket of urease revealed several important binding interactions.

Keywords: Urease Inhibition, Molecular Structure, Thiourea, Crystal Packing.

Introduction

Urease (EC 3.5.1.5; urea amidohydrolase) is a nickel-containing metalloenzyme that converts urea into ammonia and carbon dioxide by hydrolysis [1]. The presence of urease has extensively been reported in a variety of organisms, comprising fungi, bacteria, algae, including plants [2]. The virulence influence of the bacterial ureases was found to be a significant factor in the development of numerous harmful medical problems for human and animal health and also in agriculture [3]. The involvement of bacterial ureases has also been reported in the development of stomach cancer, peptic ulcers [4] and in the formation of infectious stones [5]. The contribution of ureases has also been found in the progression of pyelonephritis, urolithiasis, hepatic coma, hepatic encephalopathy, and urinary catheter encrustation [6].

Urease inhibition is used as the basis of early treatment of infections of bacteria responsible for urease production. The source of large amount of ammonia production released through increased urease activity in agriculture is the main reason of economic loss and serious environmental issues [7]. As a result, the plant damage occurs primarily by deprivation of their important nutrients and secondly toxicity by ammonia, that ultimately results in rise of the soil pH [8]. These negative effects can be counterbalanced by the control of the activity of urease through the use of inhibitors. In recent years, urease inhibitors have played significant role in the treatment of the infections triggered by urease affecting bacteria [9].

In the past decades, many urease inhibitors like phosphorodiamidates, hydroxamic acid derivatives have been investigated but because of their instability or toxicity they are prevented from being employed *in vivo* and part of them had side effects. Thus, search for novel urease inhibitors with low toxicity is a significant challenge and a reasonable demand [10] and thus the quest for the search of these analogues with the ability to inhibit urease for the targeted therapy development required for the treatment of urease mediated bacterial infections has been increased.

2-Nitrobenzohydrazide is an important intermediate towards the synthesis of a diverse family of heterocyclic compounds including 1,3,4-oxadiazoles [11], 1,2,4-triazoles [12], and 1,3,4-thiadiazoles [13]. These ring systems are important in modern heterocyclic chemistry, exhibiting a wide spectrum of pharmacological activities [14] such as antifungal [15], antimicrobial

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[16], anti-inflammatory [17], antitumor [18], anticonvulsants [19], antidepressants [20], and plant growth regulators [21]. In addition, they have an extensive range of therapeutic properties and are used as drugs in modern medicines. The effective nonsteroidal aromatase inhibitors, Letrozole, Vorozole, and Anastrozole, having triazole moieties, are useful for preventing breast cancer [22]. Therefore, these heterocycles are also perspective scaffolds for designing anticancer drugs.

In the present study, we have synthesized 2-nitrobenzohydrazide, which may be used to develop a variety of heterocyclic ring systems with potential biological profile. The title compound has also been evaluated for urease inhibition and the results are presented in this paper.

Experimental

Chemicals and Instrumentation

All chemicals used during reactions were of commercial grade. For TLC, Merck precoated plates (0.2 mm, DF-Alufoilien 60 F_{254}) were used. UV-light at 254 nm was used for the visualization of the product spots. Stuart melting point (SMP3) apparatus was used to record melting point. Spectrophotometer with specifications of Bio-Rad Merlin (Excalibur model), FTS 3000 MX was used for recording of IR spectra. Bruker Avance (300 MHz) spectrometer was used for recording of NMR spectra. Tetramethylsilane was used as internal standard while parts per million (ppm) is the unit used to measure the chemical shift (δ) values. The NMR spectra were recorded in DMSO-d_6 giving signal at 2.50 ppm for ^{1}H and for ^{13}C signal at 39.52 ppm. The resonance abbreviations used are: m (multiplet), d (doublet), s (singlet), Ar (aromatic). Leco CHNS-932 Elemental Analyzer from Leco Corporation (USA) was used for the elemental analysis of products.

*Synthesis of methyl 2-nitrobenzoate (**2**)*

In methanol (25 mL), 2-nitrobenzoic acid (**1**) (0.2 mol) was refluxed with catalytic amount of sulfuric acid to synthesize methyl 2-nitrobenzoate (**2**) [23, 24]. The reaction progress was monitored by TLC (thin layer chromatography). The product obtained after the completion of reaction was collected by solvent evaporation under reduced pressure. Diethyl ether (3 × 25 mL) was used for the extraction of oily product. The organic layers were washed with aqueous sodium carbonate to remove the unreacted acid. After drying with $MgSO_4$, the organic layer was filtered and evaporated to get the required ester (**2**) as an oil. Yield: 78%; R_f: 0.78 (20% EtOAc/hexane); IR (ATR, cm^{-1}): 3045 (C_{sp2}–H), 2966 (C_{sp3}–H), 1715 (C=O), 1534, 1489 (C=C), 1146 (C–O).

*Synthesis of 2-nitrobenzohydrazide (**3**)*

Methyl 2-nitrobenzoate (**2**) (0.01 mol) was converted to 2-nitrobenzohydrazide (**3**) by treating it with hydrazine hydrate (80%, 0.1 mol) under reflux in methanol [25]. The reaction progress was observed by thin layer chromatography. After completion of the reaction, the mixture was concentrated *in vacuo*. The resulting solid was filtered, washed with water to get title compound (**3**) as colorless crystals after recrystallization in ethanol. The data acquired for the compound **3** was consistent with the literature [26].

Yield: 85%; m.p.: 121–122 °C; R_f: 0.37 (10% MeOH/$CHCl_3$); IR (ATR, cm^{-1}): 3279, 3196 (NH), 1634 (C=O), 1569, 1491 (C=C), 1332, 850 (NO_2); ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.42 (s, 1H, NH), 8.03 (d, 1H, *J* = 7.8 Hz, ArH), 7.86–7.67 (m, 2H, ArH), 7.58 (d, 1H, *J* = 7.2 Hz, ArH), 4.31 (s, 2H, NH_2); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 165.2, 147.7, 144.6, 134.0, 131.5, 129.8, 124.6. Anal. Calcd. for $C_7H_7N_3O_3$ (181.05): C, 46.41; H, 3.89; N, 23.20. Found: C, 46.32; H, 3.74; N, 23.07.

X-ray Structure Determination

A colorless crystal of the synthesized hydrazide (**3**) having dimensions 0.5 × 0.25 × 0.1 mm was selected for the data collection. An Oxford SuperNova CCD diffractometer using Mo-Kα (λ = 0.71073 Å) X-radiation at 130 K was used for collection of diffraction data. The direct methods were used to solve the structure and refined by full-matrix least squares using SHELX-97 [27]. With the exception of those hydrogen atoms bonded to nitrogen, all other hydrogen atoms were refined in idealized positions.

Urease Inhibition Assay

Reaction mixtures consisting of an enzyme solution (25 µL) (*Jack bean* urease) and buffers (55 µL) having 100 mM urea were incubated at 30 °C with the test synthesized compound (5 µL) (0.5 mM concentration) for 15 min in 96-well plates. The indophenol method was used for the determination of urease activity by measuring ammonia production as defined by Weatherburn [28].

Concisely, 45 µL each of phenol reagent (0.005% w/v sodium nitroprusside and 1% w/v phenol)

and 70 µL of alkali reagent (0.1% active chloride NaOCl and 0.5% w/v NaOH) were added to each well. A microplate reader (Molecular Device, USA) was used to measure the increasing absorbance at 630 nm after 50 min. All the reactions were done in triplicate in a final volume of 200 µL. The soft Max Pro software (Molecular Device, USA), was used for processing the results (change in absorbance per min). Thiourea was used as a standard inhibitor of urease. The whole assays were performed at pH 6.8. The formula used to calculate the percentage inhibitions was:

$$\%\text{inhibition} = [100-(OD_{testwell}/OD_{control}) \times 100]$$

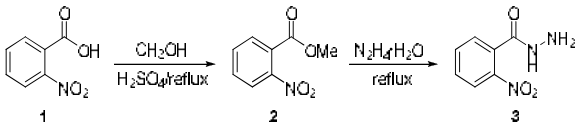
Molecular Docking Protocol

The molecular construction of the compound was performed using ChemBioDraw Ultra 14 suite (PerkinElmer Inc.) and converted into 3D conformations by ChemBio3D [29]. Optimized ligand was docked into the active site of the protein using Autodock 4.0 [30] with default parameters. Crystal structure of the enzyme, urease (PDB: 3LA4) [31] used for protein-ligand interactions, was retrieved from Protein Data Bank [32]. The target protein was prepared by the addition of hydrogen, removal of water and the removal of co-crystallized ligands. All other parameters were used with the default settings. The active site of the enzyme was used as possible binding sites to analyze the potential binding of isolated compound. 2D and 3D images were taken using discovery studio visulizer.

Results and Discussion

Chemistry

The synthetic route adopted for the compound, 2-nitrobenzohydrazide (**3**), is illustrated and outlined in Scheme-1. Commercial 2-nitrobenzoic acid was converted into its methyl ester by refluxing in methanol according to the literature procedure [23, 24]. The ester (**2**) was characterized by the appearance of stretching vibrations for C_{sp3}–H (2966), C=O_{ester} (1715) and C–O (1146) cm^{-1} and disappearance of typical broad peak of acidic (OH) in the range of 3400–2400 cm^{-1}. Treatment of methyl 2-nitrobenzoate (**2**) with hydrazine monohydrate in methanol afforded the desired hydrazide (**3**) in 85% yield [25]. The IR spectrum of hydrazide exhibited the characteristic stretching band for primary NH_2 at 3279 cm^{-1} along with a shoulder, while absorption band for secondary NH was observed at 3196 cm^{-1}. The title compound was also confirmed by a shift in carbonyl stretching absorption from 1715 cm^{-1} in ester (**2**) to 1634 cm^{-1} in the hydrazide (**3**). In ^{1}H NMR spectrum, two characteristic broad singlets for NH and NH_2 protons at 10.42 and 4.31 ppm, respectively, confirmed the formation of hydrazide. In ^{13}C-NMR, appearance of amidic carbonyl at 165.23 ppm also confirmed the formation of the title compound.



Scheme-1: Synthetic protocol of 2-nitrobenzohydrazide (**3**).

X-ray Crystallography

The molecular structure was further confirmed by single crystal X-ray diffraction analysis. Experimental details are given in Table-1 while selected bond distances, angles and dihedral angles are given in Table-2 along with H-bond geometries in Table-3. A thermal ellipsoid plot for compound (**3**) is illustrated in Fig. 1. All bond distances are within the expected ranges, the two nitrogens of the hydrazide moiety adopt two different geometries as expected; the amide-type nitrogen (N2) is planar which optimised the resonance interaction with the carbonyl group (C7–O1), while the amino-like nitrogen (N3) is pyramidalised, a geometry that optimises both H-bond donor and H-bond acceptor properties of N3.

Table-1: Crystal data and structure refinement details for compound **3**.

Formula weight	**181.16**
Empirical formula	**$C_7H_7N_3O_3$**
Wavelength	**0.7107 Å**
Temperature	**130.0(1) K**
Space group	**P 21 21 21**
Crystal system	**Orthorhombic**
Unit cell dimensions	***a* = 4.9764(4) Å**
	***b* = 12.5280(3) Å**
	***c* = 12.8512(11) Å**
	$\alpha = \beta = \gamma = 90°$
Z	**4**
Volume	**801.20(19) $Å^3$**
Density (calculated)	**1.502 Mg/m^3**
Absorption coefficient	**0.120 mm^{-1}**
Crystal size	**0.5 × 0.25 × 0.1 mm^3**
F(000)	**376**
Theta range for data collection	**3.17 to 28.92°**
Index ranges	**–6<=h<=6, –10<=k<=15, –11<=l<=17**
Reflections collected	**2857**
Independent reflections	**1676 [R(int) = 0.0262]**
Completeness to theta = 25.00°	**99.9%**
Absorption correction	**Semi-empirical from equivalents**
Max. and min. transmission	**1.00000 and 0.94533**
Refinement method	**Full-matrix least-squares on F^2**
Data / restraints / parameters	**1676 / 0 / 130**
Goodness-of-fit on F^2	**1.063**
Final R indices [I>2sigma(I)]	**R_1 = 0.0388, wR_2 = 0.0816**
R indices (all data)	**R_1 = 0.0484, wR_2 = 0.0896**
Largest diff. peak and hole	**0.190 and -0.262 e.$Å^{-3}$**

Table-2: Bond lengths [Å] and angles [°] for compound **3**.

Bond Length [Å]			
C(1)-C(6)	1.383(3)	C(1)-C(2)	1.394(3)
C(1)-C(7)	1.498(3)	C(2)-C(3)	1.382(3)
C(2)-N(1)	1.469(3)	C(3)-C(4)	1.384(3)
C(4)-C(5)	1.376(3)	C(5)-C(6)	1.392(3)
C(7)-O(1)	1.230(2)	C(7)-N(2)	1.329(3)
N(1)-O(3)	1.225(2)	N(1)-O(2)	1.223(2)
N(2)-N(3)	1.415(2)	N(2)-H(2)	0.89(3)
N(3)-H(3A)	0.91(2)	N(3)-H(3B)	0.90(2)
Bond Angle (°)			
C(6)-C(1)-C(2)	117.32(18)	C(6)-C(1)-C(7)	122.09(17)
C(2)-C(1)-C(7)	120.21(17)	C(3)-C(2)-C(1)	123.03(19)
C(3)-C(2)-N(1)	117.28(18)	C(1)-C(2)-N(1)	119.57(18)
C(2)-C(3)-C(4)	118.10(19)	C(2)-C(3)-H(3)	121.0
C(4)-C(3)-H(3)	121.0	C(5)-C(4)-C(3)	120.3(2)
C(5)-C(4)-H(4)	119.8	C(3)-C(4)-H(4)	119.8
C(4)-C(5)-C(6)	120.6(2)	C(4)-C(5)-H(5)	119.7
C(6)-C(5)-H(5)	119.7	C(5)-C(6)-C(1)	120.5(2)
O(1)-C(7)-N(2)	123.39(19)	O(1)-C(7)-C(1)	120.96(18)
N(2)-C(7)-C(1)	115.64(17)	O(3)-N(1)-O(2)	123.91(18)
O(3)-N(1)-C(2)	117.49(17)	O(2)-N(1)-C(2)	118.55(18)
C(7)-N(2)-N(3)	121.74(17)	C(7)-N(2)-H(2)	123.7(15)
N(3)-N(2)-H(2)	114.6(15)	N(2)-N(3)-H(3A)	110.2(14)
N(2)-N(3)-H(3B)	108.8(13)	H(3A)-N(3)-H(3B)	106.2(19)

Table-3: Hydrogen bonds for compound **3** [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
N(2)-H(2)...O(1)#1	0.89(3)	1.95(3)	2.813(2)	161(2)
N(3)-H(3A)...O(2)#2	0.91(2)	2.34(2)	3.128(2)	145(2)
N(3)-H(3B)...N(3)#3	0.90(2)	2.19(2)	3.053(2)	162(2)

Symmetry transformations used to generate equivalent atoms:
#1 x-1,y,z #2 -x+2,y-1/2,-z+1/2 #3 x+1/2,-y+1/2,-z

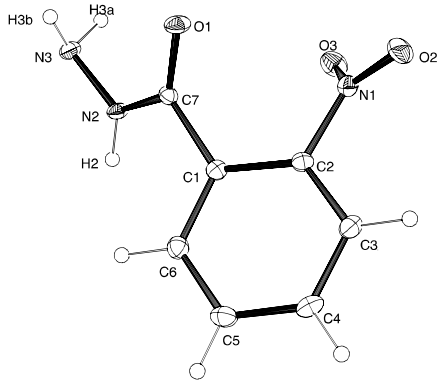


Fig. 1: A thermal ellipsoid plot for compound **3**.

The crystal packing of **3** features a combination of N–H…N and N–H…O hydrogen bonds which form H-bonded ribbons extending down the x-direction, stabilised by N(2)-H(2)...O(1) and N(3)-H(3B)...N(3) hydrogen bonds (Fig. 2). Partial packing diagram showing the hydrogen chains extending along the y direction is represented in Fig. 3.

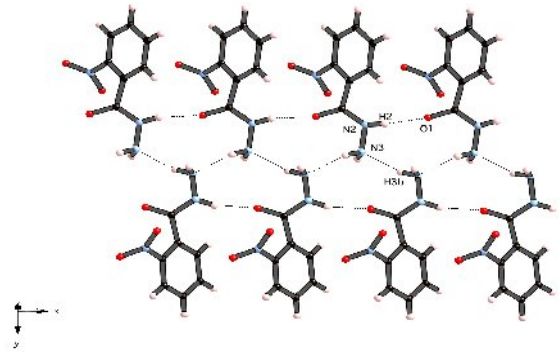


Fig. 2: Partial crystal packing diagram showing the H-bonded ribbons of compound 3 extending along the x direction.

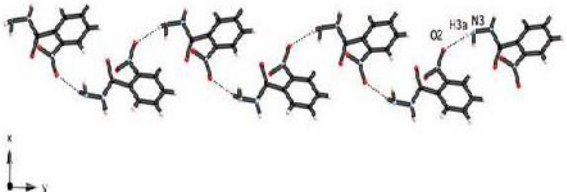


Fig. 3: Partial packing diagram showing the hydrogen chains extending along the y direction.

Urease Inhibition

The newly synthesized compound **3** was screened for urease inhibitory potency by measuring the production of ammonia employing the indophenol method of Weatherburn [28], and the results (Table-4) revealed that compound showed excellent activity (IC_{50} = 4.25 ± 0.08 µM), higher than the standard thiourea (IC_{50} = 21.00 ± 0.11 µM). The electron-withdrawing nature of aryl ring as well as free N–H for hydrogen bonding to the enzyme could be the vital factors for good urease inhibition results.

Table-4: Urease inhibition activity and Gibb's free binding energy of synthesized compound (**3**).

Compound	Conc. (mM)	IC_{50} ± SEM [µM][a,b]	Gibb's free energy
3	**0.5**	**4.25 ± 0.08**	**–6.62**
Thiourea	**0.5**	**21.00 ± 0.11**	**–0.89**

[a]Experiments performed in triplicate
[b]SEM = standard error of the mean

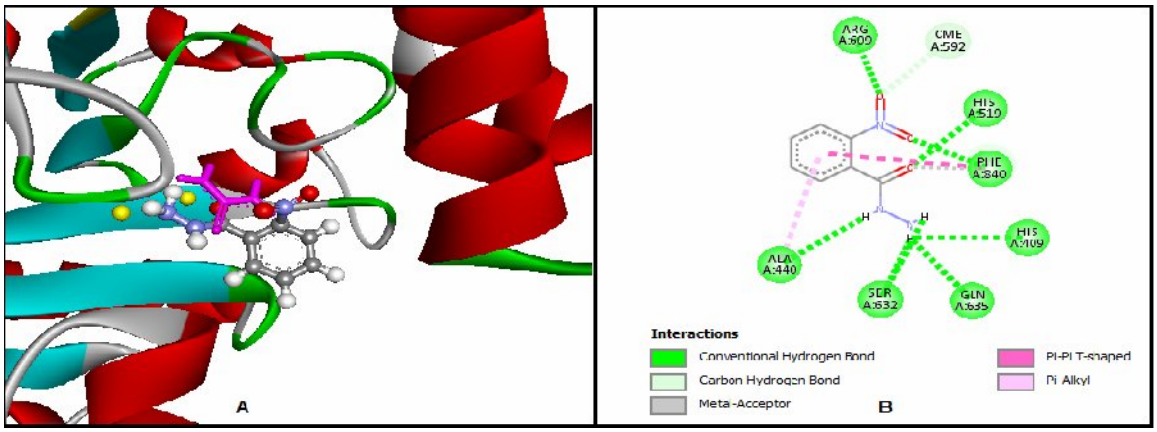


Fig. 4: (a) 3D binding pose representation of compound 3 and thiourea (pink) in the active pocket of urease; (b) 2D interaction diagram of **3**.

Molecular docking studies

The molecular docking analysis of the synthesized compound **3** against urease enzyme was performed using AutoDock that provided Gibb's free binding energy of –6.62 Kcal.mol^{-1} as shown in Table-4. The binding interactions explicitly revealed that ligand is docked in the active site of the protein with strong binding interactions including conventional hydrogen bonds with Arg80, His519, Phe840, His409, Gln635, Ser632 and Ala 440, in addition to metal interaction with Nickel through carbonyl oxygen as represented in Fig. 4b. Other interactions including pi-pi stacking with Phe840 and pi-alkyl interaction with Ala440 were also observed (Fig. 4b). 3D binding pose representation of compound **3** against urease compared to thiourea (pink) is represented in Fig. 4a. In general, strong binding interactions of the ligand with protein complemented by good binding affinity expressed by binding energy propose this ligand as a possible candidate of urease inhibitor.

Conclusion

In this study, synthesis of 2-nitrobenzohydrazide, an important intermediate for a variety of biologically active heterocycles, is reported. The title compound is characterized by spectroscopic techniques and unequivocally confirmed by X-ray diffraction analysis. The compound **3** was screened against urease enzyme. The results revealed that the synthesized structure is a potent inhibitor of urease with an IC_{50} value of 4.25 ± 0.08 µM. Molecular docking analysis of the 2-nitrobenzohydrazide revealed the possible binding modes inside the active pocket of urease. Further studies towards the synthesis of a library of other analogues leading to the construction of a large variety of heterocyclic cores for potential biological applications are underway in our laboratory.

Acknowledgements

Generous support from Higher Education Commission of Pakistan (project No. 21-978/SRGP/R&D/HEC/2016) is gratefully acknowledged.

Supplementary data

Crystallographic data for the structure revealed in this study has been deposited with the Cambridge Crystallographic Center, **CCDC: 864118**. Copies of these data could be accessed free of charge on application to The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44(0)1223-336033; e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk.

References

1. N. E. Dixon, T. C. Gazzola, *Jack bean* urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel?, *J. Am. Chem. Soc.,* **97**, 4131 (1975).
2. R. L. Blakeley, B. Zerner, Jack bean urease: the first nickle enzyme, *J. Mol. Catal.,* **23**, 263 (1984).
3. H. L. T. Mobley, M. D. Island, R. P. Hausinger, Molecular biology of microbial ureases, *Microbiol. Rev.* **59**, 451 (1995).
4. C. Montecucco, R. Rappuoli, Living dangerously: how Helicobacter pylori survives in the human stomach, *Nat. Rev. Mol. Cell Biol.,* **2**, 457 (2001).
5. R. P. Hausinger, P. A. Karplus, "in Handbook of Metalloproteins," (A. Messerschmidt, R. Hubber, T. Poulos, K. Wieghardt, Ed.), John Wiley & Sons: Chichester, UK, p. 867 (2001).
6. H. L. Mobley, R. P. Hausinger, Microbial ureases: significance, regulation, and molecular characterization, *Microbiol. Rev.,* **53**, 85 (1989).

7. K. M. Khan, A. Wadood, M. Ali, Z. Ullah, Z.U. Haq, M. A. Lodhi, M. Khan, S. Perveen M. I. Choudhary, Identification of potent urease inhibitors *via* ligand- and structure-based virtual screening and *in vitro* assays *J. Mol. Graph. Modell.,* **28**, 792 (2010).
8. J. M. Bremenr, Recent research on problems in the use of urea as nitrogen fertilizer, *Fert. Res.,* **42**, 321 (1995).
9. B. Krajewska, Ureases I. Functional, catalytic and kinetic properties: A review, *J. Mol. Catal. B: Enzym.,* **59**, 9 (2009).
10. W. J. Mao, P. C. Lv, L. Shi, H. Q. Li, H. L. Zhu, Synthesis, molecular docking and biological evaluation of metronidazole derivatives as potent Helicobacter pylori urease inhibitors, *Bioorg. Med. Chem.,* **17**, 7531 (2009).
11. I. Khan, A. Ibrar, N. Abbas, Oxadiazoles as Privileged Motifs for Promising Anticancer Leads: Recent Advances and Future Prospects, *Arch. Pharm. Chem. Life Sci.,* **347**, 1 (2014).
12. M. H. Khan, S. Hameed, K. A. Yasin,T. Akhtar, K. M. Khan, Design, synthesis, and urease inhibition studies of a series of 4-amino-5-aryl-3H-1, 2, 4-triazole-3-thiones, *Monatsh. Chem.,* **141**, 479 (2010).
13. I. Khan, S. Ali, S. Hameed, N. H. Rama, M. T. Hussain, A. Wadood, R. Uddin, Z. U. Haq, A. Khan, S. Ali, M. I. Choudhary, Synthesis, antioxidant activities and urease inhibition of some new 1,2,4-triazole and 1,3,4-thiadiazole derivatives, *Eur. J. Med. Chem.,* **45**, 5200 (2010).
14. A. Kumar, D. Bansal, K. Bajaj, S. Sharma, K. Archana, V. K. Srivastava, Synthesis of some newer derivatives of 2-amino benzoic acid as potent anti-inflammatory and analgesic agents, *Bioorg. Med. Chem.,* **11**, 5281 (2003).
15. G. Turan-Zitoui, Z. A. Kaplancikli, M. T. Yildiz, P. Chevallet, D. Kaya, Synthesis and antimicrobial activity of 4-phenyl/cyclohexyl-5-(1-phenoxyethyl)-3-[*N*-(2-thiazolyl)acetamido]thio-4H-1,2,4-triazole derivatives, *Eur. J. Med. Chem.,* **40**, 607 (2005).
16. N. Demirbas, S. A. Karaoglu, A. Demirbas, K. Sancak, Synthesis and antimicrobial activities of some new 1-(5-phenylamino-[1,3,4]thiadiazol-2-yl)methyl-5-oxo-[1,2,4]triazole and 1-(4-phenyl-5-thioxo-[1,2,4]triazol-3-yl)methyl-5-oxo-[1,2,4]triazole derivatives, *Eur. J. Med. Chem.,* **39**, 793 (2004).
17. M. Amir, K. Shikha, Synthesis and anti-inflammatory, analgesic, ulcerogenic and lipid peroxidation activities of some new 2-[(2,6-dichloroanilino) phenyl]acetic acid derivatives, *Eur. J. Med. Chem.,* **39**, 535 (2004).
18. R. Romagnoli, P. G. Baraldi, O. Cruz-Lopez, C. L. Cara, M. D. Carrion, A. Brancale, E. Hamel, L. Chen, R. Bortolozzi, G. Basso, G. Viola, Synthesis and antitumor activity of 1,5-disubstituted 1,2,4-triazoles as cis-restricted combretastatin analogues, *J. Med. Chem.,* **53**, 4248 (2010).
19. X. S. Cui, J. Chen, K. Y. Chai, J. S. Lee, Z. S. Quan, Synthesis and anticonvulsant evaluation of 3-substituted-4-(4-hexyloxyphenyl)-4*H*-1,2,4-triazoles, *Med. Chem. Res.,* **18**, 49 (2009).
20. R. Kharb, P. C. Sharma, M. S. Yar, Pharmacological significance of triazole scaffold, *J. Enzyme Inhib. Med. Chem.,* **21**, 1 (2011).
21. R. Ustabas, D. Ünlüer, G. Kör, Crystal Structure 4-(2,4-Dihydroxybenzylideneamino)-5-methyl-2H-1,2,4-triazol-3(4H)-one, *J. Chem. Crystallogr.,* **41**, 1237 (2011).
22. P. E. Goss, K. Strasser-Weippl, Aromatase inhibitors for chemoprevention, *Best Pract. Res. Clin. Endocrinol. Metab.,* **18**, 113 (2004).
23. I. Khan, A. Ibrar, J. M. White, Ethyl 2,6-Dimethoxybenzoate: Synthesis, Spectroscopic and X-ray Crystallographic Analysis, *Crystals*, **2**, 521 (2012).
24. M. Hanif, I. Khan, N. H. Rama, S. Noreen, M. I. Choudhary, P. G. Jones, M. Iqbal, Synthesis, crystal structure and *β*-glucuronidase inhibition activity of some new hydrazinecarboxamides and their 1,2,4-triazole derivatives, *Med. Chem. Res.,* **21**, 3885 (2012).
25. I. Khan, M. Hanif, M. T. Hussain, A. A. Khan, M. A. S. Aslam, N. H. Rama, J. Iqbal, Synthesis, Acetylcholinesterase and Alkaline Phosphatase Inhibition of Some New 1,2,4-Triazole and 1,3,4-Thiadiazole Derivatives*, Aust. J. Chem.,* **65**, 1413 (2012).
26. S. Sunder, N. P. Peet, R. J. Barbuch, A reinvestigation of the cyclodesulfurization of thiosemicarbazides, *J. Heterocycl. Chem.,* **18**, 1601 (1981).
27. G. M. Sheldrick, *Acta Crystallogr.,* **A64**, 112 (2008).
28. M. W. Weatherburn, Phenol-hypochlorite reaction for determination of ammonia, *Anal. Chem.,* **39**, 971 (1967).
29. N. Mills. "ChemDraw Ultra 10.0". *J. Am. Chem. Soc*., **128**, 13649 (2006).
30. G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility, *J. computational Chem.,* **30**, 2785 (2009).
31. A. Balasubramanian, K. Ponnuraj, Crystal structure of the first plant urease from Jack Bean: 83 years of journey from its first crystal to molecular structure, *J. Mol. Biol*., **400**, 274 (2010).
32. www.rcsb.org. H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne. The Protein Data Bank. *Nucleic Acids Res*., **28**, 235 (2000).