Articles

Synthesis and Biological Activities of Some New 3,6-Disubstituted 1,2,4-Triazolo[3,4-b]1,3,4-thiadiazole Derivatives

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A series of aromatic hydrazides **3a-j** were prepared by refluxing esters **2a-j** with hydrazine hydrate in methanol, which were prepared by the esterification of **1a-j**. Acetohydrazides **3a-j** upon treatment with carbon disulfide and methanolic potassium hydroxide yielded potassium dithiocarbazate salts **4a-j**, which on refluxing with hydrazine hydrate yielded substituted 4-amino-5-aryl-3*H*-1,2,4-triazole-3-thiones **5a-j**. The target compounds **6a-j** were synthesized by condensing furan-3-carboxylic acid in the presence of polyphosphoric acid under reflux. The structures of newly synthesized compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, elemental analysis and mass spectrometric studies. All the synthesized compounds were screened for their urease, acetylcholine esterase inhibition, antioxidant and alkaline phosphatase inhibition activity. Almost all of the compounds **6a-j** showed good to excellent activities against urease and acetylcholine esterase more than the reference drugs. Compounds **6f** and **6g** were more potent scavenger of free radicals than the reference *n*-propyl gallate. Compound **6b** and **6h** showed excellent activities of alkaline phosphatase as compare to the reference KH_2PO_4.

Key Words : Triazolothiadiazoles, Urease, Acetylcholine esterase, Antioxidant, Antibacterial activity

Introduction

Urease (urea amidohydrolase, E.C. 3.5.1.5) is an enzyme that catalyzes hydrolysis of urea to ammonia and carbamate, which is the final step of nitrogen metabolism in living organisms.[1,2] Carbamate decomposes rapidly and spontaneously, yielding a second molecule of ammonia. These reactions may cause significant increase in pH and ammonium ions (NH_4^+) and is responsible for negative effects of urease activity in human health and agriculture. Urease is responsible for urinary tract and gastrointestinal infections,[3] possibly causing severe diseases such as peptic ulcers and stomach cancer as in the case of *Helicobacter pylori*.[4] Ureases are also involved in the development of urolithiasis, pyelonephritis, hepatic encephalopathy, hepatic coma, and urinary catheter encrustation.[5] The efficiency of soil nitrogen fertilization with urea (the most used fertilizer worldwide) decreases due to ammonia volatilization and root damage caused by soil pH increased.[6] Control of the activity of urease through the use of inhibitors could counteract these negative effects.

Acetylcholinesterase is a serine hydrolase (AChE, acetylcholine hydrolase, E.C. 3.1.1.7) that plays an essential role in the cholinergic synapses. Hydrolysis of the neurotransmitter acetylcholine (ACh) in the nervous system by acetyl-
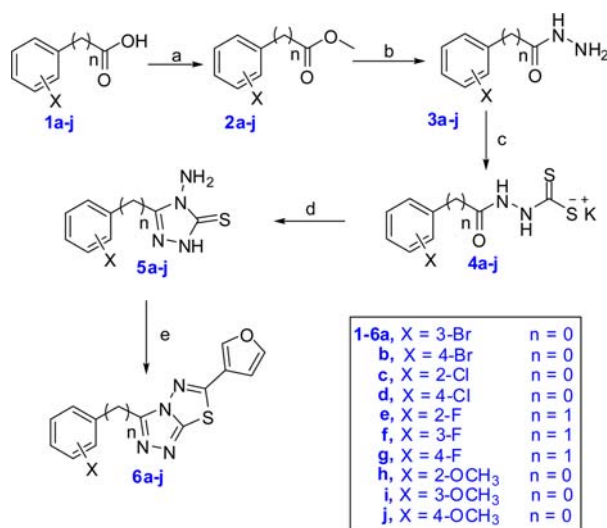
cholinesterase is known to be one of the most efficient enzyme catalytic reactions. The basis of this high efficiency has been sought by means of ligand-binding studies using various substrates and has led to the suggestion that the active center is composed of a cationic esteratic subsite containing the active serine, an anionic site which accommodates the choline moiety of ACh and a peripheral anionic site (PAS).[7,8] The primary physiologic role of the AChE peripheral site is to accelerate the hydrolysis of acetylcholine at low substrate concentrations.[9,10]

The role of cholinergic system has been an intensive issue of interest in Alzheimer disease, which is a neurodegenerative disorder causing deterioration of memory and other cognitive functions.[11,12] In Alzheimer's disease, a cholinergic deficiency in the brain has been reported.[13,14] Therefore the synthesis and study of inhibitors of acetylcholinesterase may aid to the development of therapeutically useful compounds to treat such neurological disorders. Acetylcholinesterase inhibitors donepezil hydrochloride, galantamine hydrobromide and rivastigmine tartrate are the current approved drugs for the treatment of Alzheimer patients.[15] However, acetylcholinesterase inhibitors present some limitations, such as their short half-lives and excessive side effects caused by activation of peripheral cholinergic systems, as well as hepatotoxicity, which is the most frequent and important

side effects of these drug therapies.[16-18] For this reason, alternative and complementary therapies need to be developed.

Reactive oxygen species (ROS), capable of causing damage to DNA, has been associated with carcinogenesis, coronary heart disease, and many other health problems related to advancing age.[19,20] In low concentrations, synthetic antioxidants are also in use for many industrial processes *e.g.* inhibition of radical formation for preventing premature polymerization during processing, storage and transportation of unsaturated monomers. They exert their effects by scavenging or preventing the generation of ROS[21] which can protect the formation of free radicals and retard the progress of many chronic diseases[22] including cancer, neurodegenerative, inflammation and cardiovascular diseases.[23]

Alkaline phosphatase (ALP, E.C. 3.1.3.1.) is a non-specific phosphomonoester hydrolase that catalyzes the hydrolysis and transphosphorylation of a wide variety of organic monophosphates and regulates the functions of many biological systems.[24-26] The widespread occurrence of ALP in nature suggests its involvement in fundamental biochemical processes, however, there is no positive evidence regarding its physiological function, or the nature of the natural substrates. Hydrolysis of phosphoesters, phosphate transferase activity, protein phosphatase activity, phosphate transport, modulation of organic cation transport, and involvement in cell proliferation have been suggested as possible functions of ALP.[27,28] The biological action of the alkaline phosphatase in serum is associated with metabolic bone (Hypophosphatasia) and liver diseases and also is used as a marker of osteoblastic differentiation.[29] Alkaline phosphatases may potentially be employed as therapeutic agents and therapeutic targets and show several uses in clinical medicine and in biotechnology.

Results and Discussion

Synthesis of 3,6-Disubstituted 1,2,4-Triazolo[3,4-*b*][1,3,4]thiadiazoles 6a-j. Formation of 3,6-disubstituted 1,2,4-triazolo[3,4-*b*]1,3,4-thiadiazole **6a-j** were indicated by IR spectrum by appearance of peak due to C-S-C stretching vibrations in the range of 987-1070 cm^{-1} and disappearance of signal due to C=S stretching vibration in the range of 1315-1354 cm^{-1} and N-H stretching vibration in the range of 3115-3220 cm^{-1}, respectively. ^{1}H NMR and ^{13}C NMR confirmed the formation of triazolothiadiazoles **6a-j**. Further confirmations of triazolothiadiazoles **6a-j** were carried out by mass spectral data and elemental analysis. Adapted route for the synthesis of triazolothiadiazoles **6a-j** are outlined in Scheme 1.

Pharmacology.

Urease Inhibition: Almost all the compounds showed excellent urease inhibition more than the reference thiourea as determined through the standard urease assay. Thiourea with IC$_{50}$ value of 26 ± 5 µM and K$_i$ value of 21 ± 5 µM was used as reference drug. Compound **6j** with IC$_{50}$ value of 0.764 ± 0.03 µM and K$_i$ value of 0.450 ± 0.06 was most active compound of the series. Compound **6c** with IC$_{50}$ value of 32.72 ± 0.12 µM and K$_i$ value of 22.41 ± 0.43 µM was least active. All other compounds with IC$_{50}$ values ranging from 1.55 ± 0.16 to 9.11 ± 1.7 µM showed potent activity more than the reference thiourea. Most active compound **6j** has 4-methoxyphenyl group attached with rigid triazolothiadiazole moiety. Least active compound **6c** has 2-chlorophenyl group attached with fused triazolothiadiazole ring. Urease inhibition activities of the compounds **6a-j** are given in the Table 1. Some of the compound got precipitated in the assay media, they were not evaluated for their urease inhibition assay. We are further investigating these compounds.

Acetylcholine Esterase Inhibition Activity: All of the synthesized target molecules were screened for their acetylcholine esterase inhibition activities. Neostigmine methyl sulfate with IC$_{50}$ value of 69.1 ± 8.2 µM and Donepezil with IC$_{50}$ value of 0.021 ± 0.004 µM were used as reference drug. Almost all of the compounds showed more activity than

Scheme 1. Synthesis of 3,6-disubstituted 1,2,4-triazolo[3,4-*b*] [1,3,4]thiadiazoles **6a-j**.
Reagents and conditions: (a) H$_2$SO$_4$ (conc.), methanol, reflux, 5-6 h; (b) Hydrazine hydrate, methanol, reflux, 8-10 h; (c) Carbon disulfide, potassium hydroxide, methanol, stirring at 0-5 °C, 1-2 h; (d) Hydrazine hydrate, reflux, 10-12 h; (e) Polyphosphoric acid, furan-3-carboxylic acid, reflux, 4-5 h.

Table 1. Urease inhibition activities (IC$_{50}$, and K$_i$) of compounds **6a-j**

Compounds	IC$_{50}$ (µM)	K$_i$ (µM)
Thiourea	26 ± 5	21 ± 5
6a	7.92 ± 0.97	4.15 ± 0.65
6b	3.12 ± 0.25	1.85 ± 0.04
6c	32.72 ± 0.12	22.41 ± 0.43
6d	NS	ND
6e	9.11 ± 1.7	5.25 ± 1.1
6f	2.19 ± 0.05	1.16 ± 0.024
6g	1.55 ± 0.16	8.96 ± 1.3
6h	NS	ND
6i	5.51 ± 1.2	3.18 ± 0.67
6j	0.764 ± 0.03	0.450 ± 0.06

NS = Not soluble, ND = Not determined

Table 2. Acetylcholine esterase inhibition and K_i values of compounds **6a-j**

Compounds	IC_{50}^{a} (µM) ± SEM or (% Inhibition)^{b}	K_i (µM)
Neostigmine methylsulfate	69.1 ± 8.2^{a}	63.1 ± 7.1
Donepezil	0.021 ± 0.004	0.019 ± 0.004
6a	0.344 ± 0.012^{a}	0.251 ± 0.021
6b	5.31 ± 0.42^{a}	6.91 ± 0.37
6c	78.21 ± 0.43^{a}	65 ± 54
6d	8.65 ± 0.25^{a}	4.65 ± 0.76
6e	$(19 \pm 3)^{b}$	ND
6f	NS	ND
6g	NS	ND
6h	1.98 ± 0.75^{a}	1.54 ± 0.65
6i	4.76 ± 0.14^{a}	4.34 ± 0.13
6j	1.78 ± 0.16^{a}	1.62 ± 0.14

NS = Not soluble, ND = Not determined. ^{a}The IC_{50} is the concentration at which 50% of the enzyme activity is inhibited. ^{b}The % inhibition of the enzyme activity caused by 1 mM of the tested compounds, given in parentheses

Table 4. Alkaline phosphatase inhibition activities of the synthesized compounds **6a-j**

Compounds	IC_{50}^{a} (µM) ± SEM or (% Inhibition)^{b}	K_i (µM)
KH_2PO_4	3.11 ± 0.03^{a}	1.5
6a	6.7 ± 5.7^{a}	4.92 ± 4.61
6b	0.061 ± 0.001^{a}	0.044 ± 0.001
6c	10 ± 2^{b}	7.3 ± 2
6d	71 ± 8^{b}	52 ± 7
6e	NS	ND
6f	NS	ND
6g	54 ± 5^{b}	52 ± 7
6h	0.15 ± 0.02^{a}	0.11 ± 0.02
6i	20 ± 2^{b}	14 ± 2
6j	NS	ND

NS = Not soluble, ND = Not determined. ^{a}The IC_{50} is the concentration at which 50% of the enzyme activity is inhibited. ^{b}The % inhibition of the enzyme activity caused by 1 mM of the tested compounds

Neostigmine methyl sulfate. Most active compound **6a** with IC_{50} value of 0.344 ±0.012 µM showed comparable activity to that of Donepezil reference drug. All other compounds showed IC_{50} value ranging from 1.78 ± 0.16 µM to 78.21 ± 0.43. It is clear from the Table 2 that acetylcholine esterase inhibition activity is highly dependent on substituent X. Most active compound **6a** has 3-bromo group as substituent X while least active compound **6c** has 2-chloro group as substituent X. Compounds **6e**, **6f** and **6g** having fluoro-benzyl substituent attached with triazolothiadiazole were not compatible with the assay media. These compounds got precipitated in the reference assay media and IC_{50} values of these compounds were not determined. IC_{50} and K_i values of target compounds **6a-j** are given in the Table 2.

Antioxidant: In the DPPH free radical scavenging assay, *n*-propyl gallate with IC_{50} value 40.8 µM was used as reference drug. Compound **6f** with IC_{50} value of 31.54 µM was the most active compound of the series. Compound **6f** has 3-fluorobenzyl group as substituent attached with tri-

Table 3. DPPH radical scavenging activities of the synthesized compounds **6a-j**

Compounds	% RSA	IC_{50} (µM)
n-propyl gallate	92.5	40.8
6a	56.54	196
6b	50.65	276
6c	87.21	40.41
6d	45.81	360
6e	50.98	252
6f	93.83	31.54
6g	94.21	39.32
6h	29.22	412
6i	31.5	422
6j	65.29	56

azolothiadiazole ring, showing greater electron donating ability. Compound **6i** with IC_{50} value 422 µM was the least active compound and has 3-methoxy phenyl group as substituent. All other compound showed moderate to good activities with IC_{50} value ranging from 40.41 to 412 µM. IC_{50} values and percentage radical scavenging assays (% RSA) are given in the Table 3.

Alkaline Phosphatase: Potassium dihydrogen phosphate with IC_{50} value of 3.11 ± 0.03 µM was used as standard drug for alkaline phosphatase inhibition assay. Compound **6b** with IC_{50} value of 0.061 ± 0.001 µM was the most active compound of the series. It has 4-bromophenyl phenyl group as substituent attached with triaozolothiadiazole ring. Compound **6d** with percent inhibition of 71 ± 8% was the least active compound of the series. All other compound showed IC_{50} values ranging from 0.15 ± 0.02 µM to 6.7 ± 5.7 µM. Some of the compounds precipitated in the assay media and their IC_{50} value was not determined and the percent inhibition of **6c**, **6d**, **6g** and **6i** was here reported as in Table 4.

Experimental

All the common solvents and chemicals were of analytical grade or dry distilled. The qualitative analysis of the synthesized compounds were ascertained by thin layer chromatography and the R_f values were determined by employing pre-coated silica gel aluminium plates, Kieslgel 60 F_{254} from Merck (Germany), using petroleum ether:ethyl acetate (8:2) as an eluent and TLC was visualized under UV lamp. Melting points were determined on a Stuart melting point apparatus (SMP3) and are uncorrected. The IR spectra were recorded on Bruker Optics Alpha FT-IR spectrophotometer. NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer with TMS as an internal standard. The multiplicities were expressed as s = singlet, d = doublet, t = triplet, q = quartet, dt = doublet of triplets. Molecular mass was

determined using mass spectrometer with ESI (positive) probe and ethyl acetate was used as solvent. Samples were diluted with LCMS grade methanol and filtered through PTFE membrane filter (0.45 μm pore size) before injecting them using direct syringe pump. Elemental analysis was performed on Leco CHNS-932 Elemental Analyzer, Leco Corporation (USA). The R_f values were determined on pre-coated silica gel aluminium plates, Kieslgel 60 F_{254} Merck (Germany), using petroleum ether:ethyl acetate (8:2) as an eluent and chromatograms were visualized by UV at 254 and 365 nm.

Synthesis of Substituted Aromatic Esters 2a-j and Aromatic Acid Hydrazides 3a-j. Substituted aromatic acid **1a-j** were esterified by refluxing in methanol and in the presence of catalytic amount of sulfuric acids. Substituted aromatic esters were converted into their corresponding acid hydrazides by refluxing in hydrazine hydrate in methanol through literature procedure.[30,31]

Synthesis of 3-Substituted 4-Amino-5-aryl-3*H*-1,2,4-triazole-3-thiones 5a-j. Potassium hydroxide (0.125 mol) was dissolved in dry methanol (50 mL). To the solution, aryl acid hydrazide **3a-j** (0.125 mol) was added and cooled the solution in ice. To this, carbon disulfide (0.125 mol) was added in small portions with constant stirring. The solid product of potassium dithiocarbazate **4a-j** was formed, filtered, washed with chilled diethyl ether, dried and was taken in water (20 mL) and hydrazine hydrate (0.250 mol) was added, and followed by refluxed for 10-12 h. The reaction mixture turned to green with evolution of hydrogen sulfide and finally it became homogeneous. It was then poured in ice and acidified with 37% hydrochloric acid. The white precipitates was filtered, washed with cold water and recrystallized from aqueous methanol.

4-Amino-3-(3-bromophenyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5a): White solid; yield: 76%; mp 200-202 °C; R_f 0.82 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3320, 3161 (NH), 3071 (sp^2 CH), 1640 (C=N), 1574, 1511 (C=C), 1315 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 14.04 (s, 1H, NH), 8.20 (s, 1H, Ar-H), 8.01 (d, 1H, J = 7.8 Hz, Ar-H), 7.75-7.71 (m, 1H, Ar-H), 7.51-7.47 (m, 1H, Ar-H), 5.80 (s, 2H, NH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 178.33, 167.71, 136.35, 130.54, 129.16, 127.41, 127.07, 124.77; ESI/MS for C$_8$H$_7$BrN$_4$S (m/z, + ion mode) 270.96 (M+H); Anal. Calcd. for C$_8$H$_7$BrN$_4$S: C, 35.44; H, 2.60; N, 20.66; S, 11.83; Found: C, 35.83; H, 2.45; N, 20.17; S, 11.76.

4-Amino-3-(4-bromophenyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5b): White solid; yield: 75%; mp 173-174 °C; R_f 0.55 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3295, 3082 (NH), 3028 (sp^2 CH), 1605 (C=N), 1588-1456 (C=C), 1325 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 14.13 (s, 1H, NH), 8.28-8.23 (m, 2H, Ar-H), 7.70-7.66 (m, 2H, Ar-H), 5.76 (s, 2H, NH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 162.60, 157.91, 137.03, 127.71, 126.97, 120.19; GC-ESI/MS for C$_8$H$_7$BrN$_4$S (m/z, + ion mode): 270.97 (M+H); Anal. Calcd. for C$_8$H$_7$BrN$_4$S: C, 35.44; H, 2.60; N, 20.66; S, 11.83; Found: C, 36.12; H, 3.01; N, 20.75; S, 12.24.

4-Amino-3-(2-chlorophenyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5c): White solid; yield: 71%; mp 208-209 °C; R_f 0.87 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3315, 3175 (NH), 3033 (sp^2 CH), 1610 (C=N), 1577-1506 (C=C), 1354 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 13.99 (s, 1H, NH), 7.56-7.51 (m, 1H, Ar-H), 7.50-7.47 (m, 3H, Ar-H), 5.52 (s, 2H, NH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 167.31, 150.23, 149.42, 132.70, 133.91, 133.11, 130.17, 125.70; ESI/MS for C$_8$H$_7$ClN$_4$S (m/z, + ion mode): 227.04 (M+H); Anal. Calcd. for C$_8$H$_7$ClN$_4$S: C, 42.39; H, 3.11; N, 24.72; S, 14.15; Found: C, 42.47; H, 2.98; N, 25.11; S, 13.45.

4-Amino-3-(4-chlorophenyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5d): Light brown solid; yield: 74%; mp 190-192 °C; R_f 0.67 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3273, 3209 (NH), 3073 (sp^2 CH), 1619 (C=N), 1572-1502 (C=C), 1333 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 12.66 (s, 1H, NH), 7.56-7.51 (m, 2H, Ar-H), 7.47-7.43 (m, 2H, Ar-H), 5.79 (s, 2H, NH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 167.17, 153.21, 149.18, 135.80, 130.87, 122.04; ESI/MS for C$_8$H$_7$ClN$_4$S (m/z, + ion mode): 227.04 (M+H); Anal. Calcd. for C$_8$H$_7$ClN$_4$S: C, 42.39; H, 3.11; N, 24.72; S, 14.15; Found: C, 41.86; H, 4.34; N, 24.11; S, 15.13.

4-Amino-3-(2-fluorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5e): Brown solid; yield: 72%; mp 197-199 °C; R_f 0.61 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3266, 3124 (NH), 3028 (sp^2 CH), 1598 (C=N), 1586-1497 (C=C), 1337 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 14.24 (s, 1H, NH), 8.32-8.27 (m, 1H, Ar-*H*), 7.94-7.89 (m, 1H, Ar-H), 7.68-7.63 (m, 1H, Ar-H), 7.54-7.49 (m, 1H, Ar-H), 5.36 (s, 2H, NH$_2$), 4.65 (s, 2H, CH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.11, 167.54, 152.98, 134.23, 132.17, 131.11, 129.65, 127.48, 124.10; ESI/MS for C$_9$H$_9$FN$_4$S (m/z, + ion mode): 225.11 (M+H); Anal. Calcd. for C$_9$H$_9$FN$_4$S: C, 48.20; H, 4.05; N, 24.98; S, 14.30; Found: C, 49.10; H, 3.35; N, 25.81; S, 14.71.

4-Amino-3-(3-fluorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5f): Yellowish brown solid; yield: 70%; mp 232-234 °C; R_f 0.57 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3275, 3025 (NH), 3038 (sp^2 CH), 1587 (C=N), 1577-1514 (C=C), 1347 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 14.01 (s, 1H, NH), 8.50-8.46 (m, 1H, Ar-*H*), 8.01-7.97 (m, 1H, Ar-H), 7.75-7.71 (m, 1H, Ar-H), 7.51-7.47 (m, 1H, Ar-H), 5.45 (s, 2H, NH$_2$), 4.32 (s, 2H, CH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.23, 167.17, 153.11, 133.14, 132.87, 130.52, 129.27, 127.43, 124.18; ESI/MS for C$_9$H$_9$FN$_4$S (m/z, + ion mode): 225.09 (M+H); Anal. Calcd. for C$_9$H$_9$FN$_4$S: C, 48.20; H, 4.05; N, 24.98; S, 14.30; Found: C, 48.81; H, 3.95; N, 25.21; S, 14.11.

4-Amino-3-(4-fluorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5g): Yellow solid; yield: 70%; mp 232-234 °C; R_f 0.57 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3289, 3044 (NH), 3038 (sp^2 CH), 1587 (C=N), 1577-1514 (C=C), 1341 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 13.86 (s, 1H, NH), 7.58-7.52 (m, 2H, Ar-H), 7.43-7.38 (m, 2H, Ar-H), 5.61 (s, 2H, NH$_2$), 4.19 (s, 2H, CH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.54, 166.89, 152.87, 132.16, 130.56, 128.36, 123.68; ESI/MS for C$_9$H$_9$FN$_4$S (m/z, + ion mode): 225.04 (M+H); Anal. Calcd. for C$_9$H$_9$FN$_4$S: C, 48.20; H, 4.05; N, 24.98; S, 14.30; Found: C, 49.21; H, 3.59; N, 26.11; S,

13.98.

4-Amino-3-(2-methoxyphenyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5h): Brown solid; yield: 70%; mp 240-242 °C; R_f 0.69 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3288, 3154 (NH), 2918 (sp^2 CH), 1592 (C=N), 1526-1497 (C=C), 1342 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 14.03 (s, 1H, NH), 7.43-7.39 (m, 1H, Ar-H), 7.34-7.29 (m, 3H, Ar-H), 5.49 (s, 2H, NH$_2$), 3.68 (s, 3H, OCH$_3$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 168.36, 162.37, 159.36, 135.69, 134.26, 133.52, 130.62, 119.45, 56.73; ESI/MS for C$_9$H$_{10}$N$_4$OS (*m/z*, + ion mode): 223.10 (M+H); Anal. Calcd. for C$_9$H$_{10}$N$_4$OS: C, 48.63; H, 4.53; N, 25.21; S, 14.43; Found: C, 49.18; H, 4.89; N, 26.09; S, 14.54.

4-Amino-3-(3-methoxyphenyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5i): Brown solid; yield: 70%; mp 240-242 °C; R_f 0.69 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3288, 3154 (NH), 2918 (sp^2 CH), 1592 (C=N), 1526-1497 (C=C), 1337 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 13.86 (s, 1H, NH), 8.12 (s, 1H, Ar-H), 7.84 (d, 1H, *J* = 7.8 Hz, Ar-H), 7.56-7.48 (m, 1H, Ar-H), 7.39-7.34 (m, 1H, Ar-H), 5.74 (s, 2H, NH$_2$), 3.63 (s, 3H, OCH$_3$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 169.27, 163.25, 160.03, 135.22, 135.01, 133.40, 131.58, 120.23, 56.27; ESI/MS for C$_9$H$_{10}$N$_4$OS (*m/z*, + ion mode): 223.12 (M+H); Anal. Calcd. for C$_9$H$_{10}$N$_4$OS: C, 48.63; H, 4.53; N, 25.21; S, 14.43; Found: C, 49.25; H, 4.34; N, 26.13; S, 14.87.

4-Amino-3-(4-methoxyphenyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (5j): Light brown solid; yield: 75 %; mp 215-217 °C; R_f 0.71 (petroleum ether:ethyl acetate, 7:3) 3294, 3130 (NH), 2937 (sp^2 CH), 1585 (C=N), 1561-1506 (C=C), 1335 (C=S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 13.81 (s, 1H, NH), 7.98-7.95 (m, 2H, Ar-H), 7.07-6.97 (m, 2H, Ar-H), 5.76 (s, 2H, NH$_2$), 3.72 (s, 3H, OCH$_3$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 167.12, 161.31, 158.22, 132.57, 130.11, 118.61, 56.32; ESI/MS for C$_9$H$_{10}$N$_4$OS (*m/z*, + ion mode): 223.12 (M+H); Anal. Calcd. for C$_9$H$_{10}$N$_4$OS: C, 48.63; H, 4.53; N, 25.21; S, 14.43; Found: C, 48.22; H, 4.83; N, 26.10; S, 14.23.

Synthesis of 3,6-Disubstituted 1,2,4-Triazolo[3,4-*b*][1,3,4]thiadiazoles 6a-f. A mixture of 3-substituted 4-amino-5-aryl-3*H*-1,2,4-triazolo-3-thiones **5a-j** (1 mmol) and furan-3-carboxylic acid (1.1 mmol) in polyphosphoric acid (5-6 mL) was refluxed for 4-5 h. The reaction mixture was slowly poured in crushed ice with stirring and neutralized with sodium hydrogen carbonate. Solid material was filtered, washed with cold water and dried to furnish triazolothiadiazole as colored solid.

3-(3-Bromophenyl)-6-(furan-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6a): Yellow solid; yield: 68%; mp 235-237 °C; R_f 0.72 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3084 (sp^2 CH), 2922-2847 (sp^3 CH), 1598 (C=N), 1587-1468 (C=C), 988 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.56 (s, 1H, Ar-H), 8.30 (d, 1H, *J* = 7.8 Hz, Ar-*H*), 8.10 (s, 1H, Ar-H), 7.62-7.56 (m, 1H, Ar-H), 7.49 (dd, 1H, *J* = 7.5, 2.3 Hz, Ar-H), 7.41-7.38 (m, 1H, Ar-H), 6.95-6.90 (s, 1H, Ar-H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 158.95, 154.87, 151.78, 145.23, 143.50, 133.35, 130.56, 129.16, 127.44, 124.77,

123.07, 118.79, 113.56; ESI/MS for C$_{13}$H$_7$BrN$_4$OS (*m/z*, + ion mode): 347.01 (M+H); Anal. Calcd. for C$_{13}$H$_7$BrN$_4$OS: C, 44.97; H, 2.03; N, 16.14; S, 9.24; Found: C, 44.70; H, 1.98; N, 15.92; S, 9.11.

3-(4-Bromophenyl)-6-(furan-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6b). Yellow solid; yield: 75%; mp 242-244 °C; R_f 0.80 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3107 (sp^2 CH), 2917-2832 (sp^3 CH), 1597 (C=N), 1557-1480 (C=C), 1070 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.26-8.21 (m, 2H, Ar-H), 8.10 (s, 1H, Ar-H), 7.70-7.63 (m, 2H, Ar-H), 7.64-7.57 (m, 1H, Ar-H), 6.95-6.89 (m, 1H, Ar-H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 158.76, 145.58, 144.98, 144.18, 143.25, 132.28, 130.23, 124.47, 118.23, 115.22, 107.56; ESI/MS for C$_{13}$H$_7$BrN$_4$OS (*m/z*, + ion mode): 346.98 (M+H); Anal. Calcd. for C$_{13}$H$_7$BrN$_4$OS: C, 44.97; H, 2.03; N, 16.14; S, 9.24; Found: C, 44.91; H, 2.01; N, 16.12; S, 9.21.

3-(2-Chlorophenyl)-6-(furan-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6c): Yellow solid; yield: 69%; mp 232-234 °C; R_f 0.74 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3103 (sp^2 CH), 2921-2845 (sp^3 CH), 1600 (C=N), 1588-1470 (C=C), 1054 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.01 (s, 1H, Ar-H), 7.78-7.72 (m, 1H, Ar-H), 7.51-7.47 (m, 4H, Ar-H), 6.86 (m, 1H, Ar-H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 159.21, 145.26, 145.02, 143.36, 134.02, 132.01, 131.91, 130.47, 127.02, 124.98, 124.11, 117.22, 113.56; ESI/MS for C$_{13}$H$_7$ClN$_4$OS (*m/z*, + ion mode): 303.09 (M+H); Anal. Calcd. for C$_{13}$H$_7$ClN$_4$OS: C, 51.58; H, 2.33; N, 18.51; S, 10.59; Found: C, 51.42; H, 2.21; N, 18.41; S, 10.51.

3-(4-Chlorophenyl)-6-(furan-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6d): White solid; yield: 70%; mp 200-202 °C; R_f 0.76 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3056 (sp^2 CH), 2899-2811 (sp^3 CH), 1597 (C=N), 1588, 1456 (C=C), 1043 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.34-8.29 (m, 2H, Ar-H), 8.13 (m, 1H, Ar-H), 7.63-7.58 (m, 1H, Ar-H), 7.52-7.47 (m, 2H, Ar-H), 6.94-6.88 (m, 1H, Ar-H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 158.55, 145.18, 145.10, 144.45, 143.89, 136.56, 129.26, 127.60, 122.48, 117.82, 114.23; ESI/MS for C$_{13}$H$_7$ClN$_4$OS (*m/z*, + ion mode): 303.11 (M+H); Anal. Calcd. for C$_{13}$H$_7$ClN$_4$OS: C, 51.58; H, 2.33; N, 18.51; S, 10.59; Found: C, 51.33; H, 2.23; N, 18.31; S, 10.54.

3-(2-Fluorobenzyl)-6-(furan-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6e): Yellow solid; yield: 71%; mp 226-228 °C; R_f 0.70 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3071 (sp^2 CH), 2911-2844 (sp^3 CH), 1599 (C=N), 1588, 1456 (C=C), 989 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.34 (s, 1H, Ar-H), 8.21 (d, 1H, *J* = 7.8 Hz, Ar-H), 8.02 (s, 1H, Ar-H), 7.54-7.48 (m, 1H, Ar-H), 7.31-7.26 (m, 1H, Ar-H), 7.16-7.09 (m, 1H, Ar-H), 6.98-6.93 (m, 1H, Ar-H), 4.54 (s, 2H, CH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 159.26, 155.13, 152.01, 145.56, 144.10, 136.21, 134.02, 133.48, 130.87, 129.36, 124.32, 120.27, 108.03, 35.90; ESI/MS for C$_{14}$H$_9$FN$_4$OS (*m/z*, + ion mode): 301.16 (M+H); Anal. Calcd. for C$_{14}$H$_9$FN$_4$OS: C, 55.99; H, 3.02; N, 18.66; S, 10.68; Found: C, 56.21; H, 3.71; N, 18.20; S, 10.43.

3-(3-Fluorobenzyl)-6-(furan-3-yl)-[1,2,4]triazolo[3,4-*b*]

[1,3,4]thiadiazole (6f): Yellow solid; yield: 68%; mp 235-237 °C; R_f 0.72 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3084 (sp^2 CH), 2922-2853 (sp^3 CH), 1605 (C=N), 1597, 1468 (C=C), 987 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.56 (s, 1H, Ar-H), 8.30 (d, 1H, J = 7.8 Hz, Ar-H), 8.10 (s, 1H, Ar-H), 7.64-7.57 (m, 1H, Ar-H), 7.55-7.48 (m, 1H, Ar-H), 7.41-7.37 (m, 1H, Ar-H), 6.95-6.87 (m, 1H, Ar-H), 4.20 (s, 2H, CH$_2$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 158.95, 154.87, 151.78, 145.23, 143.50, 136.11, 134.78, 133.35, 130.56, 127.44, 123.07, 119.34, 108.36, 35.65; ESI/MS for C$_{14}$H$_9$FN$_4$OS (m/z, + ion mode): 301.11 (M+H); Anal. Calcd. for C$_{14}$H$_9$FN$_4$OS: C, 55.99; H, 3.02; N, 18.66; S, 10.68; Found: C, 56.02; H, 3.25; N, 18.35; S, 10.81.

3-(4-Fluorobenzyl)-6-(furan-3-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6g): Yellow solid; yield: 72%; mp 203-205 °C; R_f 0.78 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3124 (sp^2 CH), 2945-2856 (sp^3 CH), 1601 (C=N), 1574-1484 (C=C), 1037 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.18-8.13 (m, 2H, Ar-H), 7.83 (s, 1H, Ar-H), 7.67-7.61 (m, 2H, Ar-H), 7.48-7.43 (m, 1H, Ar-H), 6.91-6.87 (m, 1H, Ar-H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 158.21, 145.51, 143.56, 143.13, 142.87, 133.10, 130.58, 124.94, 119.61, 117.21, 114.87, 106.93; ESI/MS for C$_{14}$H$_9$FN$_4$OS (m/z, + ion mode): 301.09 (M+H); Anal. Calcd. for C$_{14}$H$_9$FN$_4$OS: C, 55.99; H, 3.02; N, 18.66; S, 10.68; Found: C, 55.87; H, 3.18; N, 18.36; S, 11.10.

6-(Furan-3-yl)-3-(2-methoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6h): Brown solid; yield: 67%; mp 226-228 °C; R_f 0.80 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3078 (sp^2 CH), 2970, 2843 (sp^3 CH), 1603 (C=N), 1598, 1457 (C=C), 1066 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.30 (s, 1H, Ar-H), 8.26 (d, 1H, J = 7.8 Hz, Ar-H), 8.13 (s, 1H, Ar-H), 7.59-7.50 (m, 2H, Ar-H), 7.36-7.29 (m, 1H, Ar-H), 7.10-7.03 (m, 1H, Ar-H), 3.66 (s, 3H, OCH$_3$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 162.31, 159.27, 154.32, 152.61, 146.89, 144.73, 136.56, 134.26, 130.52, 128.97, 127.58, 119.63, 118.23, 109.67; ESI/MS for C$_{14}$H$_{10}$N$_4$O$_2$S (m/z, + ion mode): 299.16 (M+H); Anal. Calcd. for C$_{14}$H$_{10}$N$_4$O$_2$S: C, 56.37; H, 3.38; N, 18.78; S, 10.75; Found: C, 56.89; H, 3.19; N, 19.03; S, 9.23.

6-(Furan-3-yl)-3-(3-methoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6i): Brown solid; yield: 67%; mp 226-228 °C; R_f 0.80 (petroleum ether:ethyl acetate, 7:3); IR (ν/cm^{-1}) 3078 (sp^2 CH), 2970, 2843 (sp^3 CH), 1603 (C=N), 1598, 1457 (C=C), 1069 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.52 (s, 1H, Ar-H), 8.26 (d, 1H, J = 7.8 Hz, Ar-*H*), 8.12 (s, 1H, Ar-H), 7.63-7.58 (m, 1H, Ar-H), 7.22 (dd, 1H, J = 7.5, 2.2 Hz, Ar-H), 7.26-7.18 (m, 1H, Ar-H), 7.06-6.98 (s, 1H, Ar-H), 3.28 (s, 3H, OCH$_3$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 161.20, 158.95, 154.87, 151.78, 145.10, 143.70, 136.11, 134.11, 129.64, 127.91, 127.85, 118.11, 117.26, 110.36; ESI/MS for C$_{14}$H$_{10}$N$_4$O$_2$S (m/z, + ion mode): 299.01 (M+H); Anal. Calcd. for C$_{14}$H$_{10}$N$_4$O$_2$S: C, 56.37; H, 3.38; N, 18.78; S, 10.75; Found: C, 55.78; H, 3.34; N, 18.34; S, 9.78.

6-(Furan-3-yl)-3-(4-methoxyphenyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (6j): Brown solid; yield: 67%; mp 226-228 °C; R_f 0.80 (petroleum ether:ethyl acetate, 7:3); IR (ν/

cm^{-1}) 3078 (sp^2 CH), 2970, 2843 (sp^3 CH), 1603 (C=N), 1598, 1457 (C=C), 1061 (C-S); ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.12-8.04 (m, 2H, Ar-H), 8.11 (s, 1H, Ar-H), 7.61-7.56 (m, 1H, Ar-H), 7.08-6.95 (m, 2H, Ar-H), 6.94-6.87 (m, 1H, Ar-H), 3.89 (s, 3H, OCH$_3$); ^{13}C NMR (75 MHz, CDCl$_3$) δ 161.20, 158.95, 154.87, 151.78, 145.10, 143.70, 134.11, 127.91, 127.85, 118.11, 117.26, 110.36; ESI/MS for C$_{14}$H$_{10}$N$_4$O$_2$S (m/z, + ion mode): 299.11 (M+H); Anal. Calcd. for C$_{14}$H$_{10}$N$_4$O$_2$S: C, 56.37; H, 3.38; N, 18.78; S, 10.75; Found: C, 56.98; H, 3.17; N, 19.23; S, 10.07.

Urease Inhibition Assay. The urease activity was determined by measuring amount of ammonia produced with indophenol method described by Weatherburn.[32] The assay mixture, containing 10 μL of enzyme (5 U/mL) and 10 μL of test compound in 40 μL buffer (100 mM urea, 0.01 M K$_2$HPO$_4$, 1 mM EDTA and 0.01 M LiCl, pH 8.2), were incubated for 30 min at 37 °C in 96-well plates. Briefly, 40 μL each of phenol reagents (1%, w/v phenol and 0.005%, w/v sodium nitroprusside) and 40 μL of alkali reagent (0.5%, w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The absorbance at 625 nm was measured after 30 min, using a microplate reader (Bio-Tek ELx 800^{TM}, Instruments, Inc. USA). All reactions were performed in triplicate. Percentage inhibition was calculated by using the formula 100 (OD$_{testwell}$/OD$_{control}$) × 100. Thiourea was used as the reference inhibitor of urease. The Cheng-Prusoff equation was used to calculate the K$_i$ values from the IC$_{50}$ values, determined by the non-linear curve fitting program PRISM 4.0 (GraphPad, San Diego, California, USA). At 37 °C, one μmol of ammonia produced per minute by enzyme is known as one unit of enzyme at pH 8.2.

Acetylcholine Esterase Inhibition Assay. The inhibitory activities of newly synthesized novel compounds were determined spectrophotometrically using acetylthiocholine as substrate by modifying the method of Ellman.[33] The assay solution consisted of a 20 μL of 50 mM Tris-hydrochloride buffer pH 8.0, containing (0.1 M sodium chloride and 0.02 M magnesium chloride) and 50 μL of 3 mM 5,5'-dithio-bis(2-nitrobenzoic acid). Increasing concentration of test compounds (10 μL) were added to the assay solution and pre-incubated for 15 min at 25 °C with the enzyme (10 μL). The enzymatic reaction was started by adding 10 μL of acetylthiocholine chloride as a substrate and again incubated for 5 min. The hydrolysis of acetylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitro-benzoate anion at a wavelength of 412 nm as a result of reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) with thiocholines which is cleaved by enzyme. For non-enzymatic reaction, the assays were carried out with a blank containing all components except acetylcholinesterase. The reaction rates were compared and the percent inhibition due to the presence of tested inhibitors was calculated. Neostigmine methylsulfate was used as a reference inhibitor. Each concentration was analyzed in three independent experiments run in triplicate, The Cheng-Prusoff equation was used to calculate the K$_i$ values from the IC$_{50}$ values determined by the nonlinear curve-fitting program Prism 5.0 (GraphPad,

San Diego, CA, USA).

Antioxidant Assay. The synthesized compounds were screened for antioxidant activity which is shown in the Table 4. The synthesized compounds have shown hydrogen donating ability on reaction with DPPH radical. Propyl gallate was used as reference drug. The free radical scavenging capacity of the compounds was measured by modified 1,1-diphenyl-2-picrylhydrazyl (DPPH methods described by M. I. Choudhary *et al.*[34] Test compounds were allowed to react with stable free radical, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) for half an h at 37 °C. The concentration of DPPH was kept as 100 mM. The test samples were dissolved in DMSO while the DPPH solution was prepared in ethanol. After incubation, decrease in absorption was measured at 520 nm using multiplate reader (Spectra MAX-384). Percent radical scavenging activity (RSA) of samples was determined in comparison with a DMSO treated control group[35,36] using the following formula.

$$\% \text{ RSA} = 100 - \{(\text{ODtestcompound}/\text{ODcontrol}) \times 100\}$$

Alkaline Phosphatase Inhibition Assay. Initial screening of the newly synthesized compounds was performed at a concentration of 0.1 mM of test compounds. For potentially active compounds, full concentration-inhibition curves were determined. To screen putative inhibitors, activity of calf intestinal alkaline phosphatase (CIALP) was measured by spectrophotometric assay as previously described by J. Iqbal.[37] The reaction mixture comprised of 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$ (pH 9.5), the inhibitors (0.1 mM with final DMSO 1% (v/v) and mixture was pre-incubated for 10 min by adding 5 μL of CIALP (0.025 U/mL). Then, 10 μL of substrate (0.5 mM *p*-NPP (*para* nitrophenylphosphate disodium salt) was added to initiate the reaction and the assay mixture was incubated again for 30 min at 37 °C. The change in absorbance of released *p*-nitrophenolate was monitored at 405 nm, using a 96-well microplate reader (Bio-Tek ELx 800[TM], Instruments, Inc. USA). The inhibitor activity of each sample containing the inhibitor was compared with the control sample (without inhibitor). The compounds which exhibited more than 50% inhibitory activities at calf intestinal alkaline phosphatase were further evaluated for the determination of inhibition constants. Inhibitory activities of the potent compounds were determined by a range of concentrations of inhibitors spanning 3 orders of magnitude. All the experiments were repeated three times in a triplicate manner. KH_2PO_4 was used as the reference inhibitor of calf ALP. The K_i values from the IC_{50} values determined by the nonlinear curve-fitting program Prism 5.0 (GraphPad, San Diego, CA, USA).

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