http://dx.doi.org/10.5012/bkcs.2012.33.8.2741

Synthesis, Urease and Acetylcholine Esterase Inhibition Activities of Some 1,4-Disubstituted Thiosemicarbazides and their 2,5-Disubstituted Thiadiazoles

Muhammad Saleem, Muhammad Rafiq,† Muhammad Hanif,‡ Nasim Hasan Rama,‡ Sung-Yum Seo,† and Ki-Hwan Lee*

*Department of Chemistry, Kongju National University, Kongju 314-701, Korea. *E-mail: khlee@kongju.ac.kr*
†Department of Biology, Kongju National University, Kongju 314-701, Korea
‡Department of Chemistry, Quaid-i-Azam University, Islamabad-45320, Pakistan
Received April 4, 2012, Accepted May 29, 2012

A new series of 2,5-disubstituted-1,3,4-thiadiazoles **6a-i** was synthesized by overnight stirring various 1,4-disubstituted thiosemicarbazides **5a-i** in polyphosphoric acid followed by neutralization. The structures of newly synthesized compounds **5a-i** and **6a-i** were characterized by IR, ^{1}H and ^{13}C NMR, elemental analysis and mass spectrometric studies. All the synthesized compounds were evaluated for their urease and acetylcholine esterase inhibition activities. Thiosemicarbazides **5a-i** are found to possess excellent potential for urease inhibition, more than the standard drug. Thiosemicarbazides **5a-i** are more potent urease inhibitor than their cyclic analogues thiadiazoles **6a-i**. Almost all of the compounds are excellent inhibitors of acetylcholine esterase. The inhibition of acetylcholine esterase of compounds **5a**, **5c**, **5d**, **5g**, **5i**, **6e**, **6f**, **6g**, and **6i** is much more than that of standard drug.

Key Words : Thiosemicarbazides, Thiadiazoles, Urease, Acetylcholine esterase inhibition, Potent inhibitor

Introduction

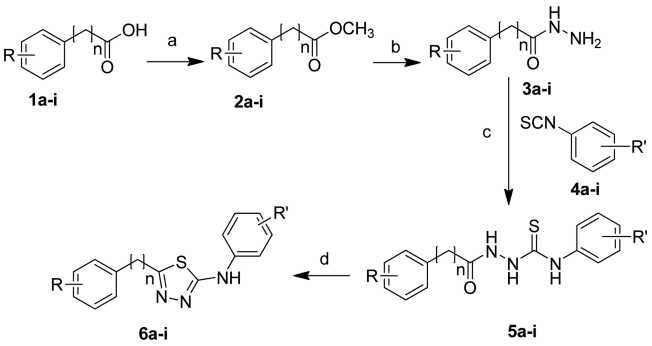
Urease (urea amidohydrolase, E.C. 3.5.1.5) is an enzyme that catalyzes hydrolysis of urea to ammonia and carbamate, which is the final step of nitrogen metabolism in living organisms.[1-3] Carbamate decomposes rapidly and spontaneously, yielding a second molecule of ammonia. These reactions may cause significant increase in pH and is responsible for negative effects of urease activity in human health and agriculture. Urease is responsible for urinary tract and gastrointestinal infections,[4] possibly causing severe diseases such as peptic ulcers and stomach cancer as in the case of *Helicobacter pylori*.[5] Ureases are also involved in the development of urolithiasis, pyelonephritis, hepatic encephalopathy, hepatic coma, and urinary catheter encrustation.[6] 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.[7] Control of the activity of urease through the use of inhibitors could counteract these negative effects.

Acetylcholinesterase is a serine hydrolase (AChE, acetylcholine hydrolase, EC 3.1.1.7) that plays an essential role in the cholinergic synapses. Hydrolysis of the neurotransmitter acetylcholine (ACh) in the nervous system by acetylcholinesterase 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).[8,9] The primary physiologic role of the AChE peripheral site is to accelerate the hydrolysis of acetylcholine at low substrate concentrations.[10,11]

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.[12,13] In Alzheimer's disease, a cholinergic deficiency in the brain has been reported.[14,15] 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.[16] 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.[16-19] For this reason, alternative and complementary therapies need to be developed.

Results and Discussion

Synthesis of Thiosemicarbazides and Thiadiazoles. Formation of thiosemicarbazides **5a-i** were indicated by IR spectrum by disappearance of characteristic broad peak of isothiocyanates (NCS) in the range of 2150-2250 cm^{-1} and appearance of the peak due to C=S group in the range of 1251-1270 cm^{-1}. Synthesis of thiosemicarbazides **5a-i** was confirmed by ^{1}H NMR by the disappearance of signals due to NH_2 hydrogens of acid hydrazides **3a-i** in the range of 4-5 ppm and appearance of three hydrogen in the range of 9.88-11.02 ppm. ^{13}C NMR further confirmed the formation of thiosemicarbazides **5a-i** by the appearance of signal due to C=S in the range of 171.56-187.01 ppm. Dehydrocyclization



Comp.	R	R'	n
6a	3-OCH_3	4-Cl	1
b	3-OCH_3	4-OCH_3	1
c	3-OCH_3	4-Cl	2
d	3-OCH_3	4-OCH_3	2
e	3,4-diOCH_3	4-Cl	0
f	3,4-diOCH_3	4-OCH_3	0
g	3-OCH_3	2-OCH_3	1
h	3-OCH_3	2-OCH_3	2
i	3,4,5-triOCH_3	3-OCH_3	2

Scheme 1. Synthesis of 2,5-disubstituted 1,3-4 thiadiazoles **6a-i:** Reagents and conditions: (a) H_2SO_4 (conc.), methanol, reflux, 8-12 h; (b) Hydrazine hydrate, methanol, reflux, 10-12 h; (c) Methanol, reflux, 10-12 h; (d) Poly phosphoric acid, stirring, 12-14 h.

of thiosemicarbazides **5a-i** were indicated by IR spectrum by the disappearance of signal due to carbonyl group in the range of 1661-1691 cm^{-1}. Formation of thiadiazoles **6a-i** were confirmed by the appearance of signal in the range of 4.84-4.90 ppm due to NH group of thiadiazole ring and disappearance of signals of NH hydrogens of thiosemicarbazides **5a-i** in the range of 9.88-11.01 ppm. Further confirmation was obtained by mass spectrometric analysis. All of the compounds showed strong molecular ion peak in mass spectra. The synthetic reactions used for the synthesis of thiosemicarbazides **5a-i** and thiadiazoles **6a-i** are outlined in Scheme 1.

Pharmacological Studies.

Urease Inhibition: The synthesized compounds were screened for their urease inhibition activity. Thiourea with IC_{50} value 26 ± 5 μM and K_i value 21 ± 5, was used as standard drug. Compound **5b** with IC_{50} value 0.849 ± 0.03 μM and K_i 0.490 ± 0.05, was most potent urease inhibitor among thiosemicarbazides **5a-i** and compound **6g** with IC_{50} value of 1.55 ± 0.16 μM and K_i value 8.96 ± 1.3 among thiadizazoles **6a-i**. Compound **5e** with IC_{50} value 9.11 ± 1.7 was least active among thiosemicarbazides **5a-i**. All other compounds showed moderate to excellent activities with IC_{50} values ranging from 1.93 ± 0.016 μM to 15 ± 4 μM. It has been observed that inhibition activity is highly dependent on substituent's R and R' of the phenyl rings. Most active compound **5b** has 3-methoxybenzyl group as R and 4-methoxyphenyl groups as R'. By replacing the 4-methoxyphenyl substituent with chlorophenyl group, urease inhibition activity decreases and IC_{50} value increases from 0.849 ± 0.03 μM to 2.25 ± 0.023 μM and by replacing the 3-methoxybenzyl group with 3-methoxyphenethyl group, again activity decreases and IC_{50} value increases from 0.849 ± 0.03 μM to 1.93 ± 0.016 μM. Urease inhibition activity also decreases by increasing the number of methoxy groups as R of the phenyl group from di-methoxy and to lesser extent in tri-methoxy phenyl substitution.

Table 1. Results of Urease inhibition activities of compounds **5a-i** and **6a-i**

Compounds code	IC_{50} (μM)	K_i
5a	2.25 ± 0.023	1.30 ± 0.01
5b	0.849 ± 0.03	0.490 ± 0.05
5c	2.08 ± 0.03	1.20 ± 0.012
5d	1.93 ± 0.016	1.11 ± 0.016
5e	9.11 ± 1.7	5.25 ± 1.1
5f	6.32 ± 1.3	4.01 ± 0.91
5g	3.11 ± 0.04	1.11 ± 0.03
5h	5.07 ± 1.1	3.22 ± 0.92
5i	4.12 ± 0.84	1.83 ± 0.07
6a	NS	ND
6b	NS	ND
6c	NS	ND
6d	5.51 ± 1.2	3.18 ± 0.67
6e	NS	ND
6f	NS	ND
6g	1.55 ± 0.16	8.96 ± 1.3
6h	15 ± 4	ND
6i	10 ± 3	ND
Thiourea	26 ± 5	21 ± 5

NS: Not soluble in the assay media, ND: Not determined

Thiosemicarbazides **5a-i** were found to be more active than thiadiazoles **6a-i**. The compounds were found inhibiting the urease in variable concentration. Urease inhibition activities of compounds **5a-i** and **6a-i** are given in Table 1.

In conclusion, thiosemicarbazides **5a-i** were found to possess excellent potential for urease inhibition, more than the standard drug. Thiosemicarbazides **5a-i** were more potent urease inhibitor than their cyclic analogues thiadiazoles **6a-i**.

Acetylcholine Esterase Inhibition: The acetylcholine esterase (E.C. 3.1.1.7 from rabbit brain) inhibition activity of the synthesized compounds **6a-i** was evaluated quantitatively by Ellman's method.[20] Compound **5c** with IC_{50} value 1.78 ± 0.16 and K_i value 1.62 ± 0.14 μM among thiosemicarbazides **5a-i** was most active and compound **6g** with IC_{50} value 0.351 ± 0.013 μM and K_i value 0.320 ± 0.012 among thiadiazoles **6a-i** was most active. Compound **5b** with % inhibition of 19 ± 3 among thiosemicarbazide **5a-i** was least active and compound **6f** with IC_{50} value 27.1 ± 4.3 μM showed minimum inhibitory activity. All other compounds showed excellent acetylcholine esterase activity with IC_{50} value ranging from 2.16 ± 0.66 μM to 27.1 ± 4.3 μM. Compound **5c** has substituent 3-methoxyphenethyl at position 3 and 2-chlorophenyl group at position 4 of thiosemicarbazide nuclei. Compound **6g** has 3-methoxy benzyl group at position 5 of thiadiazole ring. It has been observed

Table 2. Results of acetylcholine esterase inhibition activities of compounds **5a-i** and **6a-i**

Compounds code	IC_{50}[A] (μM) ± SEM[B] or (% Inhibition at 0 μM)[C]	K_i
5a	2.89 ± 0.41	2.63 ± 0.31
5b	(19 ± 3)[c]	–
5c	1.78 ± 0.16	1.62 ± 0.14
5d	4.68 ± 0.54	4.27 ± 0.44
5e	(22 ± 4)[c]	–
5f	–	–
5g	4.76 ± 0.14	4.34 ± 0.13
5h	–	–
5i	4.34 ± 0.42	3.96 ± 0.32
6a	(2 ± 0.1)[c]	–
6b	(8.4 ± 2)[c]	–
6c	(4 ± 1)[c]	–
6d	–	–
6e	2.22 ± 0.75	2.03 ± 0.55
6f	27.1 ± 4.3	24.75 ± 3.3
6g	0.351 ± 0.013	0.320 ± 0.012
6h	(14 ± 4)[c]	–
6i	2.16 ± 0.66	2.10 ± 0.48
Neostigmine methylsulfate	69.1 ± 8.2	63.1 ± 7.1

that thiadiazoles **6a-i** has more potential for acetylcholine esterase inhibition than their lower analogue **5a-i**. Results of acetylcholine esterase inhibition and K_i vaues are given in Table 2.

In conclusion, the inhibition of acetylcholine esterase of compounds **5a**, **5c**, **5d**, **5g**, **5i**, **6e**, **6f**, **6g**, and **6i** is much more than that of standard drug.

Experimental

All the common solvents and chemicals were of analytical grade or dry distilled. Reaction progress was determined by thin layer chromatographic (TLC) analysis and R_f values were determined by employing pre-coated silica gel aluminium plates, Kieslgel 60 F_{254} from Merck (Germany), using chloroform:methanol, 9:1 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. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker Avance 300 MHz spectrometer with TMS as an internal standard. Chemical shift are reported as δ values (ppm) downfield from internal tetramethylsilane of the indicated organic solution. Peak multiplicities are expressed as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; dt, doublet of triplets. Coupling constants (*J* values) are given in hertz (Hz). Mass spectra were recorded on Agilent Technologies 6890N gas chromatograph and an inert mass selective detector 5973 mass spectrometer. The elemental analysis was performed on Leco CHNS-932 Elemental Analyzer, Leco Corporation (USA). Abbreviations are used as follows: DMSO-d_6, dimethyl sulfoxide-d_6; FT-IR spectroscopy, fourier transform infrared spectroscopy.

Synthesis of Substituted Aromatic Esters 2a-i and Aromatic Acid Hydrazides 3a-i. Substituted aromatic acid **1a-i** was esterified by refluxing in methanol and in the presence of catalytic amount of sulfuric acid. Substituted aromatic ester **2a-i** was converted into their corresponding acid hydrazide **3a-i** by refluxing in hydrazine hydrate and methanol was used as solvent through reported literature procedures.[21,22]

General Procedure for the Synthesis of 1,4-Disubstituted Thiosemicarbazides 5a-i. The corresponding acid hydrazide **3a-i** (0.0068 moles) was dissolved in methanol (30 mL) and added dropwise to the solution of substituted isothiocyanate (0.0066 moles) in methanol (10 mL). The reaction mixture was refluxed for 10-12 hrs and monitored by TLC. After consumption of the starting materials, the mixture was cooled to room temperature. Evaporation of solvent under reduced pressure left crude 1,4-disubstituted thiosemicarbazide **5a-i** as an oil which was solidified on cooling.[23] It was purified by recrystallization from a mixture of ethyl acetate and petroleum ether.

4-(4-Chlorophenyl)-1-{2-(3-methoxyphenyl)acetyl}thiosemicarbazide (5a): White solid; yield: 80%; mp 156-158 ºC; R_f: 0.70 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3321, 3208 (NH), 1661 (C=O), 1595, 1557 (C=C), 1258 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.10 (aliphatic, 1H, s, N*H*-C=O), 9.74 (aliphatic, 2H, s, N*H*-C=S), 7.50 (aromatic, 2H, d, *J* = 8.7 Hz), 7.41 (aromatic, 2H, d, *J* = 8.7 Hz), 7.20 (aromatic, 1H, t, *J* = 7.8 Hz), 6.91-6.79 (aromatic, 3H, m), 3.54 (aliphatic, 3H, s), 3.40 (aliphatic, 2H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.11, 169.91, 159.61, 138.60, 137.97, 137.33, 129.65, 128.48, 122.07, 121.67, 115.40, 115.17, 55.44, 41.48. Anal. Calcd. for $C_{16}H_{16}ClN_3O_2S$: C 54.93, H 4.61, N 12.01, S 9.17; Found: C 55.21, H 4.52, N 12.12, S 9.15%.

4-(4-Methoxyphenyl)-1-{2-(3-methoxyphenyl)acetyl}-thiosemicarbazide (5b): White solid; yield: 81%; mp 149-151 ºC; R_f: 0.69 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3359, 3206 (NH), 1682 (C=O), 1593, 1542 (C=C), 1262 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.11 (aliphatic, 1H, s, N*H*-C=O), 9.50 (aliphatic, 2H, s, N*H*-C=S), 7.30-7.11 (aromatic, 3H, m), 6.99-6.72 (aromatic, 5H, m), 3.74 (aliphatic, 3H, s), 3.44 (aliphatic, 2H, s), 3.31 (aliphatic, 3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.31, 159.16, 138.60, 137.37, 136.11, 129.65, 129.01, 128.48, 125.37, 122.07, 115.39, 112.47, 55.17, 55.21, 42.75. Anal. Calcd. for $C_{17}H_{19}N_3O_3S$: C 59.11, H 5.54, N 12.17, S 9.28; Found: C 59.01, H 5.65, N 12.21, S 9.12%.

4-(4-Chlorophenyl)-1-{3-(3-methoxyphenyl)propanoyl}-thiosemicarbazide (5c): White solid; yield: 79%; mp 173-175 ºC; R_f: 0.74 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3300, 3179 (NH), 1671 (C=O), 1593, 1542 (C=C), 1252 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.94 (aliphatic, 1H, s, N*H*-C=O), 9.69 (aliphatic, 1H, s, N*H*-C=S), 9.54 (aliphatic, 1H, s, N*H*-C=S), 7.47 (aromatic, 1H, d, *J* = 8.7 Hz), 7.38 (aromatic, 1H, d, *J* = 8.7 Hz), 7.20 (aromatic, 1H,

t, J = 8.1 Hz), 7.01 (aromatic, 1H, t, J = 2.1 Hz), 6.81-6.70 (aromatic, 4H, m), 3.41 (aliphatic, 3H, s), 2.82 (aliphatic, 2H, t, J = 6.9 Hz), 2.54 (aliphatic, 2H, t, J = 6.9 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.82, 159.47, 139.89, 138.17, 137.03, 129.65, 128.52, 123.17, 121.54, 115.30, 115.11, 111.41, 55.36, 37.43, 33.24. Anal. Calcd. for $C_{17}H_{18}ClN_3O_2S$: C 56.12, H 4.99, N 11.55, S 8.81; Found: C 55.98, H 4.85, N 11.32, S 8.73%.

4-(4-Methoxyphenyl)-1-{3-(3-methoxyphenyl)propanoyl}-thiosemicarbazide (5d): White solid; yield: 77%; mp 180-182 ºC; R_f: 0.73 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3327, 3269 (NH), 1680 (C=O), 1593, 1542 (C=C), 1257 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.18 (aliphatic, 1H, s, N*H*-C=O), 9.73 (aliphatic, 2H, s, N*H*-C=S), 7.48 (aromatic, 1H, d, J = 8.4 Hz), 7.40 (aromatic, 1H, d, J = 8.4 Hz), 7.23 (aromatic, 1H, t, J = 7.8 Hz), 7.03 (aromatic, 1H, t, J = 2.1 Hz), 6.89-6.71 (aromatic, 4H, m), 3.72 (aliphatic, 3H, s), 3.69 (aliphatic, 3H, s), 2.78 (aliphatic, 2H, t, J = 6.8 Hz), 2.54 (aliphatic, 2H, t, J = 6.8 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.56, 159.61, 138.60, 137.34, 133.46, 130.71, 129.65, 128.48, 126.54, 122.07, 115.39, 112.47, 55.24, 53.37, 36.14, 34.75. Anal. Calcd. for $C_{18}H_{21}N_3O_3S$: C 60.15, H 5.89, N 11.69, S 8.92; Found: C 59.85, H 6.05, N 11.38, S 8.72%.

4-(4-Chlorophenyl)-1-(3,4-dimethoxybenzoyl)thiosemicarbazide (5e): White solid; yield: 78%; mp 165-167 ºC; R_f: 0.70 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3400, 3180 (NH), 1668 (C=O), 1596, 1510 (C=C), 1262 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.30 (aliphatic, 1H, s, N*H*-C=O), 9.61 (aliphatic, 1H, s, N*H*-C=S), 9.54 (aliphatic, 1H, s, N*H*-C=S), 7.59 (aromatic, 1H, dd, J = 8.4 Hz,1.8 Hz), 7.54 (aromatic, 1H, d, J = 1.8 Hz), 7.32 (aromatic, 2H, d, J = 9.0 Hz) 7.05 (aromatic, 1H, d, J = 8.4 Hz), 6.93 (aromatic, 2H, d, J = 9.0 Hz), 3.81 (aliphatic, 3H, s), 3.71 (aliphatic, 3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.71, 166.10, 152.19, 148.56, 137.44, 132.57, 127.99, 125.16, 121.85, 113.64, 111.62, 111.26, 53.60, 52.81. Anal. Calcd. for $C_{16}H_{16}ClN_3O_3S$: C 52.53, H 4.41, N 11.49, S 8.76; Found: C 52.75, H 4.69, N 11.77, S 8.50%.

1-(3,4-Dimethoxybenzoyl)-4-(4-methoxyphenyl)thiosemicarbazide (5f): White solid; yield: 82%; mp 174-176 ºC; R_f: 0.74 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3364, 3185 (NH), 1664 (C=O), 1599, 1511 (C=C), 1270 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.42 (aliphatic, 1H, s, N*H*-C=O), 9.83 (aliphatic, 1H, s, N*H*-C=S), 9.78 (aliphatic, 1H, s, N*H*-C=S), 7.60-7.33 (aromatic, 6H, m), 7.06 (aromatic, 1H, d, J = 8.4 Hz), 3.82 (aliphatic, 3H, s), 3.72 (aliphatic, 3H, s), 3.65 (aliphatic, 3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.42, 166.03, 152.20, 148.56, 132.57, 128.04, 125.16, 121.86, 113.66, 111.62, 111.26, 54.28, 53.32, 52.72. Anal. Calcd. for $C_{17}H_{19}N_3O_4S$: C 56.50, H 5.30, N 11.63, S 8.87; Found: C 56.22, H 5.07, N 11.48, S 8.69%.

4-(2-Methoxyphenyl)-1-{2-(3-methoxyphenyl)acetyl}thiosemicarbazide (5g): White solid; yield: 74%; mp 189-191 ºC; R_f: 0.69 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3412, 3146 (NH), 1691 (C=O), 1614, 1546 (C=C), 1258 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.37 (aliphatic, 1H, s, N*H*-C=O), 9.68 (aliphatic, 1H, s, N*H*-C=S), 9.57 (aliphatic, 1H, s, N*H*-C=S), 7.10-6.99 (aromatic, 4H, m), 7.01 (aromatic, 1H, t, J = 7.2 Hz), 6.47 (aromatic, 1H, dd, J = 8.4 Hz, 2.1 Hz), 6.41 (aromatic, 1H, dd, J = 8.4 Hz, 2.1 Hz), 6.39 (aromatic, 1H, s), 3.71 (aliphatic, s, 3H), 3.64 (aliphatic, 3H, s), 3.37 (aliphatic, 2H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.21, 165.17, 154.35, 147.51, 137.21, 133.67, 128.14, 126.34, 122.16, 114.61, 111.63, 111.03, 55.47, 53.31, 43.52. Anal. Calcd. for $C_{17}H_{19}N_3O_3S$: C 59.11, H 5.54, N 12.17, S 9.28; Found: C 59.22, H 5.66, N 12.30, S 9.45%.

4-(2-Methoxyphenyl)-1-{3-(3-methoxyphenyl)propanoyl}-thiosemicarbazide (5h): White solid; yield: 80 %; mp 186-188 ºC; R_f: 0.77 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3425, 3212 (NH), 1682 (C=O), 1612, 1585 (C=C), 1251 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.47 (aliphatic, 1H, s, N*H*-C=O), 9.83 (aliphatic, 1H, s, N*H*-C=S), 9.78 (aliphatic, 1H, s, N*H*-C=S), 7.27 (aromatic, 1H, t, J = 7.6 Hz), 7.12 (aromatic, 1H, d, J = 2.1 Hz), 7.20-6.82 (aromatic, 4H, m), 6.74 (aromatic, 1H, dd, J = 8.4 Hz, 2.4 Hz), 6.67 (aromatic, 1H, d, J = 7.8 Hz), 3.68 (aliphatic, 3H, s), 3.65 (aliphatic, 3H, s), 3.71 (aliphatic, 2H, t, J = 6.9 Hz), 3.40 (aliphatic, 2H, t, J = 6.9 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.03, 165.17, 152.56, 149.31, 137.43, 132.55, 129.34, 125.77, 121.86, 118.73, 115.24, 113.65, 111.14, 110.21, 55.72, 53.44, 42.12, 38.60. Anal. Calcd. for $C_{18}H_{21}N_3O_3S$: C 60.15, H 5.89, N 11.69, S 8.92; Found: C 59.86, H 5.69, N 11.51, S 9.14%.

4-(3-Methoxyphenyl)-1-{3-(3,4,5-trimethoxyphenyl)propanoyl}thiosemicarbazide (5i): White solid; yield: 78 %; mp 155-157 ºC; R_f: 0.69 (chloroform:methanol, 9:1); IR (ν/cm^{-1}) 3385, 3216 (NH), 1675 (C=O), 1605, 1496 (C=C), 1255 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.02 (aliphatic, 1H, s, N*H*-C=O), 9.88 (aliphatic, 1H, s, N*H*-C=S), 9.75 (aliphatic, 1H, s, N*H*-C=S), 7.44 (aromatic, 2H, s), 7.26 (aromatic, 1H, t, J = 8.1 Hz), 7.13 (aromatic, 1H, s), 7.01 (aromatic, 1H, d, J = 7.2 Hz), 6.80 (aromatic, 1H, d, J = 7.1 Hz), 3.81 (aliphatic, 9H, s), 3.72 (aliphatic, 3H, s), 3.41 (aliphatic, 2H, t, J = 6.9 Hz), 3.30 (aliphatic, 2H, t, J = 6.9 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 187.01, 167.12, 156.33, 151.23, 146.33, 138.21, 134.22, 128.23, 118.17, 114.87, 110.31, 63.78, 55.47, 43.31, 35.29. Anal. Calcd. for $C_{20}H_{25}N_3O_5S$: C 57.26, H 6.01, N 10.02, S 7.64; Found: C 57.21, H 5.89, N 10.21, S 7.41%.

General Procedure for the Synthesis of 2,5-Disubstituted 1,3,4-Thiadiazoles 6a-i. 2,5-Disubstituted-1,3,4-thiadiazoles **6a-i** was synthesized by intramolecular dehydrocyclization of various 1,4-disubstituted thiosemicarbazide derivatives **5a-i** by overnight stirring with polyphosphoric acid. Then this mixture was put on crushed ice, 2,5-disubstituted 1,3,4-thiadiazoles **6a-i** were precipitated,[24] filtered and recrystallised in ethanol.

***N*-(4-Chlorophenyl)-5-(3-methoxybenzyl)-1,3,4-thiadiazol-2-amine (6a):** White solid; yield: 76%; mp 196-198 ºC; R_f : 0.72 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3294 (NH), 1578 (C=N), 1561, 1543, 1483 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.14 (aromatic, 1H, t, J = 9.0 Hz), 7.13 (aromatic, 2H, dt, J = 9.0 Hz, 2.1 Hz), 7.01 (aromatic, 2H,

dt, J = 9.0 Hz, 2.1 Hz), 6.75 (aromatic, 1H, dd, J = 9.0 Hz, 2.1 Hz), 6.53 (aromatic, 1H, d, J = 7.5), 6.46 (aromatic, 1H, t, J = 2.1), 4.90 (NH, 1H, s, broad), 3.67 (aliphatic, 3H, s), 3.43 (aliphatic, 2H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.40, 159.62, 151.55, 136.28, 134.56, 132.93, 130.71, 129.88, 129.76, 121.25, 114.71, 113.05, 55.34, 31.88; GC-MS for $C_{16}H_{14}ClN_3OS$ (EI, *m/z,* rel. Abund. %) 331 (100), 316 (9), 298 (14), 283 (5), 121 (38), 111 (22), 91 (31), 77 (27), 51 (18); Anal. Calcd. for $C_{16}H_{14}ClN_3OS$: C, 57.91; H, 4.25; N, 12.66; S, 9.66; Found: C, 57.91; H, 4.25; N, 12.66; S, 9.66%.

5-(3-Methoxybenzyl)-*N*-(4-methoxyphenyl)-1,3,4-thiadiazol-2-amine (6b): White solid; yield: 72%; mp 165-167 ºC; R_f: 0.71 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3289 (NH), 1586 (C=N), 1574, 1517 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.12 (aromatic, 2H, dt, J = 8.4 Hz, 2.1 Hz), 7.11 (aromatic, 1H, d, J = 1.8 Hz), 7.08 (aromatic, 2H, dt, J = 9.0 Hz, 2.1 Hz), 6.72 (aromatic, 1H, dd, J = 8.4 Hz, 2.1 Hz), 6.65 (aromatic, 1H, d, J = 7.2 Hz), 6.46 (aromatic, 1H, t, J = 2.1 Hz), 4.88 (NH, 1H, s, broad), 3.80 (aliphatic, s, 3H), 3.64 (aliphatic, s, 3H), 3.33 (aliphatic, s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.64, 160.15, 154.87. 150.28, 138.91, 129.88, 129.11, 126.43, 122.20, 119.34, 114.21, 111.98, 55.78, 55.31, 31.92; GC-MS for $C_{17}H_{17}N_3O_2S$ (EI, *m/z,* rel. Abund. %) 327 (100), 312 (8), 294 (8), 279 (4), 146 (10), 121 (29), 91 (21), 77 (21), 51 (8); Anal. Calcd. for $C_{17}H_{17}N_3O_2S$: C, 62.36, H 5.23, N 12.83, S 9.79. Found: C, 62.39; H, 5.26; N, 12.77; S, 9.72%.

***N*-(4-Chlorophenyl)-5-(3-methoxyphenethyl)-1,3,4-thiadiazol-2-amine (6c):** White solid; yield: 79%; mp 177-179 ºC; R_f: 0.75 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3263 (NH), 1594 (C=N), 1534, 1523 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.62 (aromatic, 2H, dt, J = 8.7 Hz, 2.1 Hz), 7.40 (aromatic, 2H, dt, J = 6.6 Hz, 3.0 Hz), 7.15 (aromatic, 1H, t, J = 8.1 Hz), 6.74 (aromatic, 1H, dd, J = 9.3 Hz, 1.8 Hz), 6.63 (aromatic, 1H, d, J = 8.4 Hz), 6.61 (aromatic, 1H, s), 4.86 (NH, 1H, s, broad), 3.65 (aliphatic, 3H, s), 3.30-2.75 (aliphatic, 4H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.00, 159.73, 151.93, 141.96, 134.62, 132.02, 130.66, 129.95, 129.86, 120.87, 114.34, 112.18, 55.32, 37.48, 31.79; GC-MS for $C_{17}H_{16}ClN_3OS$ (EI, *m/z*, rel. Abund. %) 345 (92), 330 (4), 312 (8), 297 (2), 175 (10), 150 (6), 121 (100), 91 (65), 75 (25); Anal. Calcd. for $C_{17}H_{16}ClN_3OS$: C, 59.04; H, 4.66; N, 12.15; S, 9.27; Found: C, 59.11; H, 4.64; N, 12.17; S, 9.33%.

5-(3-Methoxyphenethyl)-*N*-(4-methoxyphenyl)-1,3,4-thiadiazol-2-amine (6d): White solid; yield: 79%; mp 202-204 ºC; R_f: 0.70 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3286 (NH), 1596 (C=N), 1544, 1515 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.27 (aromatic, 2H, dt, J = 8.7 Hz, 1.8 Hz), 7.14 (aromatic, 1H, t, J = 7.8 Hz), 7.07 (aromatic, 2H, dt, J = 9.0 Hz, 3.3 Hz), 6.73 (aromatic, 1H, dd, J = 8.7 Hz, 2.1 Hz), 6.63 (aromatic, 1H, d, J = 7.8 Hz), 6.60 (aromatic, 1H, s), 4.87 (NH, 1H, s, broad), 3.55 (aliphatic, 3H, s), 3.52 (aliphatic, 3H, s), 2.70 (aliphatic, 2H, t, J = 6.0 Hz), 2.62 (aliphatic, 2H, t, J = 6.0 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.24, 160.11, 159.72, 152.25, 142.07, 129.89, 126.58, 123.47, 120.86, 115.00, 114.33, 112.16, 55.92, 55.33, 37.41, 34.70; GC-MS for $C_{18}H_{19}N_3O_2S$ (EI, *m/z*, rel. Abund. %) 341 (100), 326 (5), 308 (6), 293 (3), 179 (12), 121 (46), 91 (26), 77 (16); Anal. Calcd. for $C_{18}H_{19}N_3O_2S$: C, 63.32; H, 5.61; N, 12.31; S, 9.39; Found: C, 63.22; H, 5.57; N, 12.41; S, 9.32%.

***N*-(4-Chlorophenyl)-5-(3,4-dimethoxyphenyl)-1,3,4-thiadiazol-2-amine (6e):** White solid; yield: 74%; mp 211-213 ºC; R_f: 0.75 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3273 (NH), 1668 (C=O), 1587 (C=N), 1534, 1512 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.60 (aromatic, 2H, dt, J = 8.7 Hz, 2.1 Hz), 7.42 (aromatic, 2H, dt, J = 8.7 Hz, 3.0 Hz), 6.95 (aromatic, 1H, d, J = 8.1 Hz), 6.87 (aromatic, 1H, dd, J = 6.1 Hz, 1.8 Hz), 6.83 (aromatic, 1H, d, J = 2.1 Hz), 4.89 (NH, 1H, s, broad), 3.65 (aliphatic, 3H, s), 3.54 (aliphatic, 3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.79, 150.83, 148.70, 137.51, 134.48, 131.29, 129.86, 126.84, 118.12, 113.71, 111.99, 111.92, 55.70, 54.23; GC-MS for $C_{16}H_{14}ClN_3O_2S$ (EI, *m/z*, rel. Abund. %) 347 (100), 332 (12), 304 (10), 183 (8), 163 (14), 120 (12), 111 (12), 75 (18); Anal. Calcd. for $C_{16}H_{14}ClN_3O_2S$: C, 55.25; H, 4.06; N, 12.08; S, 9.22; Found: C, 55.31; H, 4.14; N, 12.14; S, 9.17%.

5-(3,4-Dimethoxyphenyl)-*N*-(4-methoxyphenyl)-1,3,4-thiadiazol-2-amine (6f): White solid; yield: 77%; mp 189-191 ºC; R_f: 0.71 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3297 (NH), 1579 (C=N), 1517, 1489 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.27 (aromatic, 2H, d, J = 8.7 Hz), 7.15 (aromatic, 2H, d, J = 9.0 Hz), 7.11-6.70 (aromatic, 3H, m), 4.90 (NH, 1H, s, broad), 3.65 (aliphatic, 3H, s), 3.63 (aliphatic, 3H, s), 3.54 (aliphatic, 3H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.12, 151.00, 151.00, 150.72, 148.63, 136.52, 130.49, 127.84, 121.60, 118.42, 111.88, 111.82, 55.96, 55.93, 53.67; GC-MS for $C_{17}H_{17}N_3O_3S$ (EI, *m/z*, rel. Abund. %) 343 (100), 181 (29), 165 (7), 163 (45), 137 (15), 107 (22), 75 (17); Anal. Calcd. for $C_{17}H_{17}N_3O_3S$: C, 59.46; H, 4.99; N, 12.24; S, 9.34; Found: C, 59.52; H, 4.87; N, 12.31; S, 9.42%.

5-(3-Methoxybenzyl)-*N*-(2-methoxyphenyl)-1,3,4-thiadiazol-2-amine (6g): White solid; yield: 74%; mp 190-192 ºC; R_f: 0.69 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3225 (NH), 1591 (C=N), 1523, 1498 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.03 (aromatic, 1H, t, J = 8.7 Hz), 7.21-6.90 (aromatic, 4H, m), 6.74 (aromatic, 1H, dd, J = 8.4 Hz, 3.0 Hz), 6.46 (aromatic, 1H, d, J = 8.3 Hz), 6.39 (aromatic, 1H, t, J = 1.9 Hz), 4.85 (NH, 1H, s, broad), 3.59 (aliphatic, 3H, s), 3.54 (aliphatic, 3H, s), 2.41 (aliphatic, 2H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.63, 159.50, 154.98, 152.05, 136.39, 131.82, 130.53, 129.72, 122.10, 121.22, 120.98, 114.98, 112.95, 112.83, 55.48, 53.38, 35.91; GC-MS for $C_{17}H_{17}N_3O_2S$ (EI, *m/z*, rel. Abund. %) 327 (50), 312 (2), 294 (100), 161 (4), 146 (10), 121 (30), 91 (21), 77 (29), 51 (18); Anal. Calcd. for $C_{17}H_{17}N_3O_2S$: C, 62.36; H, 5.23; N, 12.83; S, 9.79; Found: C, 62.41; H, 5.34; N, 12.65; S, 9.78%.

5-(3-Methoxyphenethyl)-*N*-(2-methoxyphenyl)-1,3,4-thiadiazol-2-amine (6h): White solid; yield: 76%; mp 188-190 ºC; R_f: 0.74 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3286 (NH), 1586 (C=N), 1509, 1501 (C=C); ^{1}H NMR (300

MHz, DMSO-d_6) δ 7.53 (aromatic, 1H, t, J = 8.7 Hz), 7.61-7.10 (aromatic, 4H, m), 6.71 (aromatic, 2H, dd, J = 8.4 Hz, 2.9 Hz), 6.35 (aromatic, 1H, t, J = 1.9 Hz), 4.88 (NH, 1H, s, broad), 3.59 (aliphatic, 3H, s), 3.56 (aliphatic, 3H, s), 3.34 (aliphatic, 2H, t, J = 7.4 Hz), 3.31 (aliphatic, 2H, t, J = 7.4 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.31, 152.49, 142.83, 131.94, 130.72, 129.88, 122.21, 121.32, 120.88, 116.64, 114.39, 114.27, 113.29, 112.09, 111.00, 55.37, 53.44, 37.25, 34.63; GC-MS for $C_{18}H_{19}N_3O_2S$ (EI, *m/z*, rel. Abund. %) 341 (100), 326 (5), 308 (5), 293 (4), 175 (4), 121 (35), 91 (25), 77 (15); Anal. Calcd. for $C_{18}H_{19}N_3O_2S$: C, 63.32; H, 5.61; N, 12.31; S, 9.39; Found: C, 63.29; H, 5.68; N, 12.39; S, 9.26%.

***N*-(3-Methoxyphenyl)-5-(3,4,5-trimethoxyphenethyl)-1,3,4-thiadiazol-2-amine (6i):** White solid; yield: 78%; mp 203-205 ºC; R_f: 0.76 (chloroform:methanol, 9:1); IR (ν/cm^{-1}): 3200 (NH), 1595 (C=N), 1534, 1528 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.46 (aromatic, 1H, t, J = 8.1 Hz), 7.11 (aromatic, 1H, dd, J = 8.4 Hz, 2.4 Hz), 6.94 (aromatic, 1H, t, J = 1.8 Hz), 6.90 (aromatic, 1H, dt, J = 8.1 Hz, 1.6 Hz), 6.50-6.44 (aromatic, 2H, m), 4.84 (NH, 1H, s, broad), 3.69-3.65 (aliphatict, 12H, m), 3.37 (aliphatict, 2H, t, J = 7.2 Hz), 3.31 (aliphatict, 2H, t, J = 7.2 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 167.98, 151.43, 153.17, 152.06, 136.32, 136.18, 135.13, 130.65, 120.75, 115.39, 114.60, 105.89, 55.62, 54.25, 51.29, 36.82, 35.65; GC-MS for $C_{20}H_{23}N_3O_4S$ (EI, *m/z*, rel. Abund. %) 401 (38), 386 (8), 287 (13), 181 (100), 148 (12), 92 (6), 77 (6); Anal. Calcd. for $C_{20}H_{23}N_3O_4S$: C, 59.83; H, 5.77; N, 10.47; S, 7.99; Found: C, 59.81; H, 5.72; N, 10.53; S, 7.91%.

Pharmacology.

Urease Inhibition Assay: The urease activity was determined by measuring amount of ammonia being produced using indophenol method described by Weatherburn.[25] 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_2HPO_4, 1 mM EDTA and 0.01 M $LiCl_2$, pH 8.2), were incubated for 30 minutes at 37 ºC 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™, Instruments, Inc. USA). All reactions were performed in triplicate. Percentage inhibition was calculated by using the formula $100 - (OD_{testwell}/OD_{control}) \times 100$. Thiourea was used as the standard 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 being 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.[20] The assay solution consisted of a 20 μL of 50 mM Tris-hydrochloride buffer, containing 0.1 M sodium chloride, 0.02 M magnesium chloride (pH 8.0) 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. The enzymatic reaction was started by adding 10 μL of acetylthiocholinchloride 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-nitrobenzoate anion as a result of the reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) with thiocholines, catalyzed by enzymes at a wavelength of 412 nm. 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 standard 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).

Acknowledgment. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2012-0004677).

References

1. Li, M.; Ding, W.; Baruah, B.; Crans, D. C.; Wang, R. *J. Inorg. Biochem.* **2008**, *102*, 1846-1853.
2. Mobley, H. L.; Hausinger, R. P. *Microbiol. Rev.* **1989**, *53*, 85-108.
3. Karplus, P. A.; Pearson, M. A.; Hausinger, R. P. *Acc. Chem. Res.* **1997**, *30*, 330-337.
4. Collins, C. M.; D-Orazio, S. E. F. *Mol. Microbiol.* **1993**, *9*, 907-913.
5. Montecucco, C.; Rappuoli, R. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 457-466.
6. Hausinger, R. P.; Karplus, P. A. In *Handbook of Metalloproteins*; Messerschmidt, A., Hubber, R., Poulos, T., Wieghardt, K., Eds.; Wiley: Chichester, 2001.
7. Zhengping, W.; Cleemput, O. V.; Demeyer, P.; Baert, L. *Biol. Fertil. Soils* **1991**, *11*, 41-47.
8. Changeux, J. P. *Mol. Pharmacol.* **1966**, *2*, 369-392.
9. Epstein, D. J.; Berman, H. A.; Taylor, P. *Biochem.* **1979**, *18*, 4749-4754.
10. Mallender, W. D.; Szeglets, T.; Rosenberry, T. L. *J. Biol. Chem.* **1999**, *274*, 8491-8499.
11. Szeglets, T.; Mallender, W. D.; Thomas, P. J.; Rosenberry, T. L. *Biochem.* **1999**, *38*, 122-133.
12. Abramov, A. Y.; Canvari, L.; Duchen, M. R. *J. Neurosci.* **2003**, *15*, 5088-5095.
13. Butterfield, D. A. *Chem. Res. Toxicol.* **1997**, *10*, 495-506.
14. Marchbanks, R. M. *J. Neurochem.* **1982**, *39*, 9-15.
15. Taylor, P. *Pharmacological Basis of Therapeutics*; Gilman, A. G., Goodman, L. S., Murad, F., Eds.; MacMillan: New York, 1985.
16. Ellis, J. M. *J. Am. Osteopth Assoc.* **2005**, *105*, 145-158.
17. Farlow, M.; Gracon, S. I.; Hershey, L. A.; Lewis, K. W.; Sadowsky, C. H. *J. Dolan-Ureno, Jama.* **1992**, *268*, 2523-2529.
18. Lahiri, D. K.; Farlow, M. R.; Grieg, N. H.; Sambamurti, K. *Drug Dev. Res.* **2002**, *56*, 267-281.
19. Rogers, S. L.; Farlow, M. R.; Doody, R. S.; Mohs, R.; Friedhoff,

L. T. *Neurology* **1998**, *50*, 136-145.
20. Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Feather-Stone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88-95.
21. Song, C.; Xuhong, Q.; Gonghua, S.; Qingchun, H. *J. Flour. Chem.* **2002**, *117*, 63-66.
22. Hebert, N.; Hannah, A. L.; Sutton, S. C. *Tetrahedron Lett.* **1999**, *40*, 8547-8550.
23. Rehman, A.; Choudhry, M. I.; Thomsen, W. J. *Bioassay Technique for Drug Development*; Harwood Academic Publishers: The Netherland, 2001; p 22.
24. Khan, I.; Ali, S.; Hameed, S.; Rama, N. H.; Hussain, M. T.; Wadood, A.; Uddin, R.; UL-Haq, Z.; Khan, A.; Ali, S.; Choudhary, M. I. *Eur. J. Med. Chem.* **2010**, *45*, 5200-5207.
25. Weatherburn, M. W. *Anal Chem.* **1967**, *39*, 971.