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Synthesis, spectral characterization and biological activity of *S*-Substituted derivatives of 5-(4-Nitrophenyl)-1,3,4-oxadiazole-2-thiol

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ABSTRACT

A series of S-substituted derivatives of 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol (6a-l) were synthesized in various steps. Organic acid 4-nitrobenzoic acid (1) was successfully converted into ester and consequently into its hydrazide in the presence of hydrazine hydrate and methanol as a solvent. Further, 4-nitrobenzoic acid hydrazide (3) yielded 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol, on treatment with carbon disulfide in the presence of base (KOH) and ethanol. Finally the target compounds (6a-l) were obtained by stirring 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol with different electrophiles (5a-l) in the presence of sodium hydride (NaH) and dimethyl formamide (DMF). All these derivatives along with their parent compounds were characterized by IR, EI-MS and 1H-NMR spectra. These compounds were assayed for their antioxidant activities and other biological activities via screening them against acetylcholinesterase, butyrylcholinesterase and lipoxygenase enzymes, however, these showed prominent activity against acetylcholinesterase and butyrylcholinesterase enzymes.

INTRODUCTION

Substituted 1,3,4-oxadiazoles, the heterocyclic group of compounds, have been the subject of extensive concentration due to their biological and pharmacological activities [1]. 2,5-disubstituted-1,3,4-oxadiazole derivatives have attractive concentration due to wide range of biological interactions such as anti-inflammatory [2], antifungal [3,4], antiparasitic [5] and antimicrobial [6-7] effects. These derivatives have inhibitory effect against HIV replication [8]. Biological results demonstrate a very remarkable anti-tumor activity against leukemia, colon and breast cancer. 1,3,4-oxadiazoles have revealed analgesic, anti-cancer, anti-mitotic, anti depressive, anti-Parkinson and anti-tubercular activities [9-11].

Acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) consist of an enzymes family which includes serine hydrolases. The diverse specificities for the substrates and inhibitors for these enzymes are due to the differences in amino acid residues of the active sites of AChE and BChE. Actually the system of enzyme is responsible for the termination of acetylcholine at cholinergic synapses. These are key components of cholinergic brain synapses and neuromuscular junctions. The major function of AChE and BChE is to catalyze the hydrolysis of the neurotransmitter acetylcholine and termination of the nerve impulse in cholinergic synapses [12,13]. It has been found that BChE is present in appreciably higher quantity in Alzheimer's plaques than in the normal age linked dementia of brains. H_1 and H_2 receptor antagonists possess AChE inhibitory activities. Cholinesterase inhibitors raise the quantity of acetylcholine available for neuronal and neuromuscular transmission through their ability to reversibly or irreversibly. Hence, the search for new cholinesterase inhibitors is considered an important and ongoing strategy to introduce new drug candidates for the treatment of Alzheimer's disease and other related diseases [14,15].

Literature survey revealed that minor modifications in the structure of 1,3,4-oxadiazole can lead to quantitative as well as qualitative changes in the biological activity. These findings encouraged us to synthesize the various *S*-substituted derivatives of 1,3,4-oxadiazole with an objective to search new contenders of drug having significant enhanced activity and could be helpful in controlling many degenerative diseases. For this, the parent 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol (4) was prepared by converting successively 4-nitro benzoic acid into ester, hydrazide and 1,3,4-oxadiazole. Finally the title compounds were obtained by reacting 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol with different electrophiles to acquire *S*-alkyl substituted derivatives

of 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol (6a-l).

Experimental Protocol Chemistry

Procedure for the synthesis of ethyl-4-nitrobenzoate (2).

4-Nitrobenzoic acid (1) (1g, 0.0059 mol.) was taken in a round bottom flask fitted with reflux condenser. Absolute ethanol (4 mL) and conc. H_2SO_4 (0.5 mL) was added and reaction mixture was refluxed for 1 h. The completion of reaction was checked by thin layer chromatography (TLC). After completion of reaction, reaction mixture was poured into the separating funnel that contained 50 mL distilled water. Diethyl ether (15 mL) was added to the separating funnel and mixture was shaken vigorously, neutralization of reaction mixture was done by using concentrated sodium carbonate (Na_2CO_3). The solution was allowed to stand for some time, two layers were formed i.e. lower layer was aqueous and upper layer was organic that contained ethyl-4-nitrobenzoate. Lower aqueous layer was discarded and ether layer was collected through the neck of separating funnel to avoid contaminants. Diethyl ether was distilled off and pure light lemon colored ester was collected.

Procedure for the preparation of 4-nitrobenzoylhydrazide(3).

Ethyl-4-nitro benzoate (1g, 0.005 mol.) was dissolved in methanol (15 mL) in a round bottom flask simply by stirring at room temperature. After complete dissolution of compound 2, hydrazine hydrate (3.95 mL, 0.015 mol.) was added drop wise to reaction flask and set the flask mixture to stirring at room temperature for 2-3 h. Reaction completion was checked by TLC and on completion cold distilled water was added to quench the precipitates of pure product. Precipitates were filtered and washed with water.

Synthesis of 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol (4).

Compound **3** (2g, 0.011 mol.) was dissolved in ethanol (20 mL) in a 250 mL round bottom flask, potassium hydroxide (1.23g, 0.022 mol.) was added in excess to the reaction mixture, then carbon disulphide (0.66 mL, 0.011 mol) was added to the flask and reaction assembly was set to reflux for 4 h. with continuous stirring. Reaction coordinates were monitored by using TLC time by time. Color of mixture was changed from yellow to orange with the progress of reaction. After the completion of reaction distilled water was added and acidified the reaction mixture to bring its pH 2-3. Synthesized product was then filtered, washed with distilled water and recrystallized by methanol.

General procedure for the synthesis of S-substituted-5-(4-nitrophenyl)-1,3,4-oxadiazole derivatives (6a-l).

Compound (4), (0.1g, 0.0045 mol.) was dissolved in dimethyl formamide (5-10 mL) in 100 mL round bottom flask and set on stirring at room temperature, when the compound dissolved, sodium hydride (0.002g) was added as a strong base and kept on stirring for half an hour, then different electrophiles i.e. alkyl halides 5a-l were added by taking equimolar ratios to synthesize the *S*-substituted derivatives. The reaction time for different electrophiles varies from 2-3 h. Reaction completion was detected by thin layer chromatography using ethyl acetate and *n*-hexane as a solvent system. After the completion of reaction distilled water was added to the reaction mixture and precipitates were filtered and washed with water.

METHODOLOGY

Purity of the synthesized compounds was confirmed by thin layer chromatography using ethyl acetate and *n*-hexane as solvent system. Detection was made at 364 nm. Melting points were recorded by using open capillary tube method and Griffin and George melting point apparatus and were uncorrected. The IR spectra, in KBr, were recorded on a Jasco-320-A spectrophotometer. ^{1}H-NMR spectra were taken in mixture of $CDCl_3$ & CD_3OD at 500 MHz using Bruker spectrometers, demonstrating chemical shifts in ppm values. EIMS were taken by using a JMS-HX-110 spectrometer, with data system.

Spectral characterization of the synthesized compounds

5-(4-nitrophenyl)-1,3,4-oxadiazole (4):

Lemon yellow colour; yield: 75% (1.8g); M.P. 42-44°C; Molecular formula: $C_8H_5N_3SO_3$; Mol. Wt. 223; IR (KBr, cm^{-1}) ν_{max}: 3027 (C-H, stretching of aromatic ring), 2615 (S-H bond stretching), 1637 (C=N, stretching of oxadiazole ring), 1517 (C=C, aromatic stretching), 1257 (C=S bond stretching), 1241,1073 (C-O-C bond stretching), 618 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.41 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.23 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'); EIMS: *m/z* 223 (20%)[M^+], 148 (100%), 122 (35%).

2-(Ethylsulfanyl)-5-(4-nitrophenyl)-1,3,4-oxadiazole (6a):

Dirty pistachio colour; yield: 81% (0.091g); M.P. 90-91°C; Molecular formula: $C_{10}H_9N_3SO_3$; Mol. Wt. 251; IR (KBr, cm^{-1}) ν_{max}: 3033 (C-H, stretching of aromatic ring), 1630 (C=N, stretching of oxadiazole ring), 1521 (C=C, aromatic stretching), 1235,1065 (C-O-C bond stretching), 618 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.41 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.23 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 3.36 (q, 2H, H-1"), 1.51 (t, J = 7.0 Hz, 3H, H-2"); EIMS: *m/z* 251 (20%)[M^+], 222 (41%), 148 (100%), 122 (35%), 29 (75%).

2-(Propan-2-yl-sulfanyl)-5-(4-nitrophenyl)-1,3,4-oxadiazole (6b):

Buff colour; yield: 79% (0.093g); M.P. 75-76 °C; Molecular formula: $C_{11}H_{11}N_3SO_3$; Mol. Wt. 265; IR (KBr, cm^{-1}) ν_{max}: 3037 (C-H, stretching of aromatic ring), 1635 (C=N, stretching of oxadiazole ring), 1519 (C=C, aromatic stretching), 1231,1069 (C-O-C bond stretching), 613 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, □ / *ppm*): 8.40 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.22 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 3.99 (m, 1H, H-1"), 1.53 (d, J = 7.0 Hz, 6H, H-2" & H-3"); EIMS: *m/z* 265 (22%)[M^+], 222 (45%), 148 (100%), 122 (41%), 43 (75%).

2-(Prop-2-en-1-yl-sulfanyl)-5-(4-nitrophenyl)-1,3,4-oxadiazole (6c):

Mustard colour; yield: 76% (0.0897g); M.P. 96-97°C; Molecular formula: $C_{11}H_9N_3SO_3$; Mol. Wt. 263; IR (KBr, cm^{-1}) ν_{max}: 3105 (C-H, stretching of aromatic ring), 1624 (C=N, stretching of oxadiazole ring), 1525 (C=C, aromatic stretching), 1234,1072 (C-O-C bond stretching), 615 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.39 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.21 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 6.05 (m, 1H, H-2"), 5.42 (dd, J = 16.0, 1.0 Hz, 1H, H_b-3"), 5.22(dd, J = 10.0, 1.0 Hz, 1H, H_a-3"), 3.97 (br.t, J = 7.0 Hz, 2H, H-1"); EIMS: *m/z* 263 (24%)[M^+], 222 (41%), 148 (100%), 122 (47%), 41 (79%).

2-[(2-Phenylethyl)sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6d):

Lime yellow colour; yield: 86% (0.126g); M.P. 86-87°C; Molecular formula: $C_{16}H_{13}N_3SO_3$; Mol. Wt. 327; IR (KBr, cm^{-1})

ν_{max}: 3067 (C-H, stretching of aromatic ring), 1630 (C=N, stretching of oxadiazole ring), 1569 (C=C, aromatic stretching), 1234,1077 (C-O-C bond stretching), 621 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.41 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.23 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 7.27-7.14 (m, 5H, H-2" to H-6"), 3.58 (t, J = 8.0 Hz, 2H, H-8"), 3.15 (t, J = 7.0 Hz, 2H, H-7"); EIMS: *m/z* 327 (19%)[M^+], 222 (39%), 148 (100%), 122 (43%), 77 (51%), 28 (79%).

2-[(3-Phenylpropyl)sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6e):

Lemon yellow colour; yield: 79% (0.12g); M.P. 86-87°C; Molecular formula: $C_{17}H_{15}N_3SO_3$; Mol. Wt. 341; IR (KBr, cm^{-1}) ν_{max}: 3061 (C-H, stretching of aromatic ring), 1634 (C=N, stretching of oxadiazole ring), 1571(C=C, aromatic stretching), 1231,1075 (C-O-C bond stretching), 627 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.39 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.21 (br.d, J = 9.0Hz, 2H, H-2' & H-6'), 7.28-7.15 (m, 5H, H-2" to H-6"), 3.33 (t, J = 7.0 Hz, 2H, H-9"), 2.80 (t, J = 7.5 Hz, 2H, H-7"), 2.15 (t, J = 7.5 Hz, 2H, H-8"); EIMS: *m/z* 341 (21%)[M^+], 222 (41%), 148 (100%), 122 (47%), 119 (61%), 91 (73%), 77 (54%), 28 (67%).

2-(benzylsulfanyl)-5-(4-nitrophenyl)-1,3,4-oxadiazole (6f):

Light lemon yellow colour; yield: 74% (0.103g); M.P. 91-93°C; Molecular formula: $C_{15}H_{11}N_3SO_3$; Mol. Wt. 313; IR (KBr, cm^{-1}) ν_{max}: 3075 (C-H, stretching of aromatic ring), 1621 (C=N, stretching of oxadiazole ring), 1559 (C=C, aromatic stretching), 1239,1069 (C-O-C bond stretching), 631 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.41 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.21 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 7.20-7.12 (m, 5H, H-2" to H-6"), 4.61 (s, 2H, H-7"); EIMS: *m/z* 313 (20%)[M^+], 222 (51%), 148 (100%), 122 (56%), 91 (70%), 77 (44%).

2-[(2-Methylbenzyl)sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6g):

Light lemon yellow colour; yield: 76% (0.111g); M.P. 91-93°C; Molecular formula: $C_{16}H_{13}N_3SO_3$; Mol. Wt. 327; IR (KBr, cm^{-1}) ν_{max}: 3075 (C-H, stretching of aromatic ring), 1621 (C=N, stretching of oxadiazole ring),1559 (C=C, aromatic stretching), 1239,1069 (C-O-C bond stretching), 631 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.39 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.21 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 7.20-7.12 (m, 4H, H-3" to H-6"), 4.61 (s, 2H, H-7"), 2.45 (s, 3H, CH_3); EIMS: *m/z* 327 (20%)[M^+], 222 (51%), 105 (57%), 148 (100%), 122 (56%), 91 (46%), 77 (44%).

2-[(4-Flourobenzyl)sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6h):

Lemon yellow colour; yield: 76% (0.112g); M.P. 141-142°C; Molecular formula: $C_{15}H_{10}N_3SO_3F$; Mol. Wt. 331; IR (KBr, cm^{-1}) ν_{max}: 3081 (C-H, stretching of aromatic ring), 1620 (C=N, stretching of oxadiazole ring), 1593(C=C, aromatic stretching),1249,1081 (C-O-C bond stretching), 635 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.40 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.21 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 7.86 (d, J = 8.5 Hz, 2H, H-3" & H-5"), 6.95 (d, J = 8.5 Hz, 2H, H-2" & H-6"), 4.56 (s, 1H, H-7"); EIMS: *m/z* 331(26%)[M^+], 222 (49%), 109 (57%), 148 (100%), 122 (57%), 95 (48%), 77 (41%).

2-[(2-Chlorobenzyl)sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6i):

Light lemon yellow colour; yield: 78% (0.121g); M.P. 128-129°C; Molecular formula: $C_{15}H_{10}N_3SO_3Cl$; Mol. Wt. 347.5; IR (KBr, cm^{-1}) ν_{max}: 3078 (C-H, stretching of aromatic ring), 1627 (C=N, stretching of oxadiazole ring),1561 (C=C, aromatic stretching), 1251,1071 (C-O-C bond stretching), 621 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.41 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.23 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 7.45-7.27 (m, 4H, H-3" to H-6"), 4.68 (s, 1H, H-7"); EIMS: *m/z* 347 (20%)[M^+], 222 (51%), 125 (57%), 111 (46%), 148 (100%), 122 (56%), 77 (44%).

2-[(3-Chlorobenzyl)sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6j):

Lemon yellow colour; yield: 81% (0.126g); M.P. 101-102°C; Molecular formula: $C_{15}H_{10}N_3SO_3Cl$; Mol. Wt. 347.5; IR (KBr, cm^{-1}) ν_{max}: 3081 (C-H, stretching of aromatic ring), 1633 (C=N, stretching of oxadiazole ring), 1565 (C=C, aromatic stretching), 1257,1073 (C-O-C bond stretching), 637 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.40 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.21 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 7.31-7.14 (m, 4H, H-2" & H-4" to H-6"), 4.57 (s, 1H, H-7"); EIMS: *m/z* 347 (23%)[M^+], 222 (47%), 125 (53%), 111 (41%), 148 (100%), 122 (51%), 77 (42%).

2-[(4-Chlorobenzyl)sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6k):

Off white colour; yield: 79% (0.123g); M.P. 119-120°C; Molecular formula: $C_{15}H_{10}N_3SO_3Cl$; Mol. Wt. 347.5; IR (KBr, cm^{-1}) ν_{max}: 3087 (C-H, stretching of aromatic ring), 1623 (C=N, stretching of oxadiazole ring), 1597 (C=C, aromatic stretching), 1253,1085 (C-O-C bond stretching), 629 (C-S bond); ^{1}H-NMR (500 MHz, CD_3OD & $CDCl_3$, δ / *ppm*): 8.39 (br. d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.21 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 7.47 (br.d, J = 8.0 Hz, 2H, H-3" & H-5"), 7.32 (br.d, J = 8.0 Hz, 2H, H-2" & H-6"), 4.55 (s, 1H, CH_2); EIMS: *m/z* 347 (22%)[M^+], 222 (49%), 125 (57%), 111 (44%), 148 (100%), 122 (53%), 77 (45%).

2-[(4-Bromobenzyl)sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6l):

Mustard colour; yield: 83% (0.14g); M.P. 121-122 °C; Molecular formula: $C_{15}H_{10}N_3SO_3Br$; Mol. Wt. 392; IR (KBr, cm^{-1}) ν_{max}: 3077 (C-H, stretching of aromatic ring), 1621 (C=N, stretching of oxadiazole ring), 1579 (C=C, aromatic stretching), 1249,1079 (C-O-C bond stretching), 625 (C-S bond); ^{1}H-NMR (CD_3OD & $CDCl_3$, 500 MHz): δ (ppm) 8.41 (br.d, J = 9.0 Hz, 2H, H-3' & H-5'), 8.23 (br.d, J = 9.0 Hz, 2H, H-2' & H-6'), 7.48 (d, J = 8.5 Hz, 2H, H-3" & H-5"), 7.40 (d, J = 8.5 Hz, 2H, H-2" & H-6"), 4.54 (s, 1H, H-7"); EIMS: *m/z* 392 (21%)[M^+], 222 (43%), 170 (59%), 156 (61%), 148 (100%), 122 (47%), 77 (51%).

ENZYME INHIBITION ASSAYS

Acetylcholinesterase Assay

The AChE inhibition activity was performed according to the method [16] with slight modifications. Total volume of the reaction mixture was 100 µL. It contained 60 µL Na_2HPO_4 buffer with concentration of 50 mM and pH 7.7. 10 µL test compound (0.5 mM $well^{-1}$) was added, followed by the addition of 10 µL (0.005 unit $well^{-1}$) enzyme. The contents were mixed and pre-read

at 405 nm. Then contents were pre-incubated for 10 min at 37 ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM well^{-1} substrate (acetylthiocholine iodide), followed by the addition of 10 µL DTNB (0.5 mM well^{-1}). After 15 min of incubation at 37 ºC absorbance was measured at 405 nm using 96-well plate reader Synergy HT, Biotek, USA. All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM well^{-1}) was used as a positive control. The percent inhibition was calculated by the help of following equation.

$$\text{Inhibition (\%)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

IC_{50} values were calculated using EZFit Enzyme kinetics software (Perrella Scientific Inc. Amherst, USA).

Butyrylcholinesterase Assay

The BChE inhibition activity was performed according to the method [16] with slight modifications. Total volume of the reaction mixture was 100 µL containing 60 µL, Na_2HPO_4 buffer, 50 mM and pH 7.7. 10 µL test compound 0.5 mM well^{-1} was added followed by the addition of 10 µL (0.5 unit well^{-1}) BChE (Sigma Inc.). The contents were mixed and pre-read at 405 nm and then pre-incubated for 10 min at 37 ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM well^{-1} substrate (butyrylthiocholine chloride). Followed by the addition of 10 µL DTNB, 0.5 mM well^{-1}. After 15 min of incubation at 37 ºC, absorbance was measured at 405 nm using 96-well plate reader Synergy HT, Biotek, USA. All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM well^{-1}) was used as positive control. The percent inhibition was calculated by the help of following equation.

$$\text{Inhibition (\%)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

IC_{50} values were calculated using EZFit Enzyme kinetics software (Perrella Scientific Inc. Amherst, USA).

Lipoxygenase Assay

Lipoxygenase (LOX) activity was assayed according to the method [17-19] with slight modifications. A total volume of 200 µL lipoxygenase assay mixture contained 150 µL sodium phosphate buffer (100 mM, pH 8.0), 10 µL test compound and 15 µL purified lipoxygenase enzyme (600 units well^{-1},Sigma Inc.). The contents were mixed and pre-read at 234 nm and preincubated for 10 min at 25 °C. The reaction was initiated by addition of 25 µL substrate solution. The change in absorbance was observed after 6 min at 234 nm using 96-well plate reader Synergy HT, Biotek, USA. All reactions were performed in triplicates. The positive and negative controls were included in the assay. Baicalin (0.5 mM well^{-1}) was used as a positive control. The percentage inhibition (%) was calculated by formula given below.

$$\text{Inhibition (\%)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Where,

Control = Total enzyme activity without inhibitor

Test = Activity in the presence of test compound

IC_{50} values was calculated using EZFit Enzyme Kinetics software (Perrella Scientific Inc. Amherst, USA).

DPPH Assay

The stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was used for the determination of antioxidant activity. Different concentrations of compounds in respective solvents were added at an equal volume (10µl) to 90 µl of 100 µM methanolic DPPH in a total volume of 100 µl in 96-well plates. The contents were mixed and incubated at 37 °C for 30 minutes. The absorbance was measured at 517nm using Synergy HT BioTek® USA microplate reader. Quercetin and L-ascorbic acid were used as standard antioxidants. The experiments were carried out in triplicates. IC_{50} values were calculated using EZ-Fit5 Perrella Scientific Inc. Amherst USA software. The decrease in absorbance indicates increased radical scavenging activity which was determined by the following formula [20].

$$\text{Antiradical activity (\% Inhibition)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

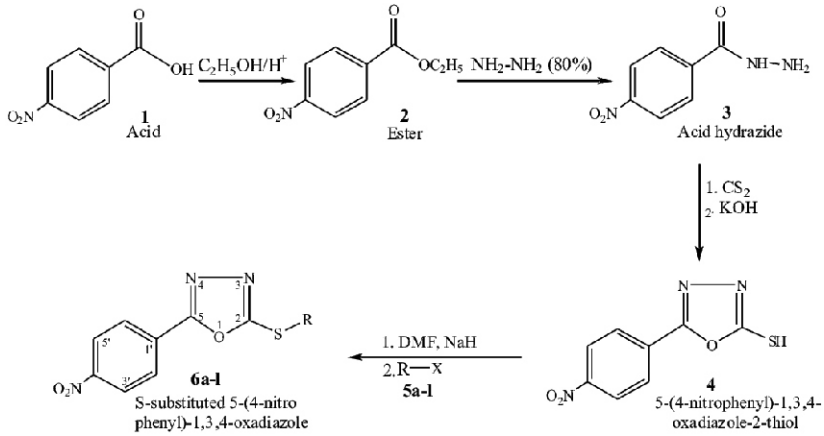
Statistical Analysis

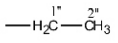
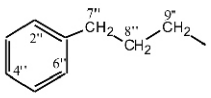
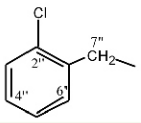
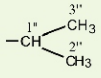
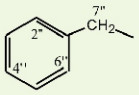
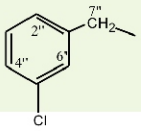
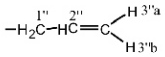
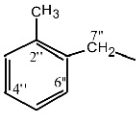
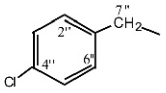
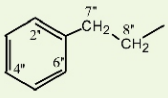
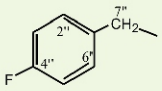
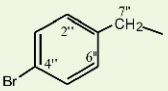
All the measurements were done in triplicate and statistical analysis was performed by Microsoft Excel 2003. Results are presented as mean ± sem.

RESULTS AND DISCUSSION

The objective of our research work was to synthesize *S*-substituted derivatives of 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol and to screen out their enzymatic activities. Parent compound 4 was synthesized by intermolecular cyclization of 4-nitrobenzoylhydrazide (3) in the presence of hydrazine hydrate and ethanol. Further, a series of *S*-substituted derivatives were brought about by treating compound 4 with different electrophiles, 5a-l, in the presence of *N, N*-dimethyl formamide and NaH which acts as a base. Reactants completely converted into products by simple stirring after 2-4 h. Precipitates were quenched by treating with cold water and recrystallized by using methanol. The compound, 6a was synthesized as light yellow colour powder having molecular formula $C_{10}H_9N_3SO_3$, established by molecular ion peak at *m/z* 251 in EI-MS and by counting the number of protons in its ^{1}H-NMR spectrum. IR spectrum showed characteristic absorption bands at 3033 cm^{-1} (C-H, stretching of aromatic ring), 1521 cm^{-1} (C=C, aromatic stretching), 1630 cm^{-1} (C=N, stretching of oxadiazole ring), 1235 cm^{-1} and 1065 cm^{-1} (C-O-C, bond stretching) confirming the presence of aromatic benzene and oxadiazole rings. EI-MS revealed two characteristic peaks m/*z* 122 and 148 after the loss of nitrophenyl and *para* nitrophenyl cyanide groups respectively. In ^{1}H-NMR spectrum signals of aromatic proton appeared at δ 8.41 ppm (br.d, J = 9.0 Hz, 2H, H-3' & H-5') and 8.23 (br.d, J = 9.0 Hz, 2H, H-2' & H-6') typical for *para* di-substituted aromatic ring. In the aliphatic region of the ^{1}H-NMR spectrum the signals resonated at 3.36 (q, 2H, H-1") and 1.51 (t, J = 7.0 Hz, 3H, H-2") which specified the ethyl group present in the molecule. On the basis of above cumulative evidences, the structure was allocated as 2-(ethylsulfanyl)-5-(4-nitrophenyl)-1,3,4-oxadiazole. During the course of this study we obtained some *S*-substituted derivatives of oxadiazole in good yield and others moderate to low. On the basis of data from IR, EIMS and ^{1}H-NMR, the structures of *S*-substituted derivatives were elucidated as described in experimental section.

The screening of these synthesized compounds against acetylcholinesterase (AchE), butyrylcholinesterase (BchE) and



Compound	R	Compound	R	Compound	R
6a		6e		6i	
6b		6f		6j	
6c		6g		6k	
6d		6h		6l	

Scheme-1: Outline for the synthesis of *S*-substituted derivatives of 5-(4-nitro phenyl)-1,3,4-oxadiazole -2-thiol

Table1: Bioactivity studies of *S*-substituted derivatives of 5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thiol.

	AChE		BChE		LOX		DPPH	
Sample Code	Inhibition (%) Conc./well (0.5 mM)	IC_{50} µM	Inhibition (%) Conc./well (0.5 mM)	IC_{50} µM	Inhibition (%) Conc./well (0.5 mM)	IC_{50} µM	Inhibition (%) Conc./well (0.5 mM)	IC_{50} µM
4	45.05±0.15	NIL	32.28±0.51	NIL	64.81±0.14	178.21±0.11	80.46±0.04	75.21±0.11
6a	69.58±0.13	164.32±0.02	58.59±0.51	163.42±0.01	54.32±0.61	<400	65.04±0.11	182.71±0.61
6b	48.55±0.71	NIL	74.06±0.82	146.51±0.61	80.97±0.15	89.41±0.11	15.78±0.75	NIL
6c	73.51±0.51	129.41±0.41	68.43±0.11	183.41±0.17	67.18±0.25	179.41±0.31	18.22±0.45	NIL
6d	61.90±0.25	188.61±0.11	72.65±0.31	158.71±0.01	61.93±0.52	252.11±0.18	15.78±0.12	NIL
6e	81.35±0.43	88.91±0.31	84.51±0.15	95.41±0.11	76.34±0.22	168.31±0.08	17.11±0.22	NIL
6f	55.02±0.21	195.61±0.61	65.93±0.43	172.91±0.63	54.75±0.63	280.11±0.18	11.28±0.13	NIL
6g	31.45±0.56	NIL	92.25±0.63	51.61±0.02	5.56±0.01	NIL	18.95±0.31	NIL
6h	80.75±0.52	70.11±0.19	79.58±0.25	147.11±0.06	68.21±0.82	219.51±0.31	17.92±0.12	NIL
6i	67.36±0.51	157.21±0.06	88.62±0.75	87.61±0.14	7.61±0.32	NIL	15.71±0.62	NIL
6j	82.44±0.61	146.21±0.14	91.31±0.14	33.81±0.63	75.21±0.31	132.21±0.61	15.63±0.71	NIL
6k	92.96±0.72	38.81±0.21	93.08±0.78	38.31±0.51	21.19±0.56	NIL	16.30±0.16	NIL
6l	94.35±0.16	40.41±0.01	94.72±0.63	52.11±0.08	40.64±0.64	NIL	17.04±0.34	NIL
Control	**Eserine 91.29±1.17**	**0.04±0.001**	**Eserine 82.82±1.09**	**0.85±0.0001**	**Baicalein 93.79±1.27**	**22.4±1.3**	**Quercetin 93.21±0.97**	**16.96±0.14**

Note: IC50 values (concentration at which there is 50% enzyme inhibition) of compounds were calculated using EZFit Enzyme kinetics software (Perella Scientific Inc. Amherst, USA).
AChE = Acetyl cholinesterase. BChE = Butyrylcholinesterase.
LOX = Lipoxygenase. DPPH = 1,1-diphenyl-2-picrylhydrazyl radical.

lipoxygenase (LOX) enzymes revealed that these molecules exhibited good inhibitory potential against acetylcholinesterase and butyrylcholinesterase as it was evident from their IC_{50} values. The results are depicted in Table-1. It is clearly evident from results that compounds 2-[(4-chlorobenzyl) sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6k), and 2-[(4-bromobenzyl) sulfanyl]-5-(4-nitrophenyl)-1,3,4-oxadiazole (6l) were found to be promising inhibitors against acetylcholinesterase enzyme having IC_{50} value of 38.81±0.21 and 40.41±0.01 µmol respectively, relative to Eserine, a reference standard with IC_{50} value of 0.04±0.001 µmol, probably due to the *S*-substitution of 4-chlorobenzyl and 4-bromobenzyl groups respectively in these molecules. The enhanced activity might be due to electron withdrawing property of chloro and bromo groups at *para* position of phenyl ring of *S*-substituted benzyl group. The screening against acetylcholinesterase enzyme exposed that the compounds 6j and 6k exhibited good inhibitory potential having IC_{50} value of 33.81±0.63 and 38.31±0.51 µmol as compared to standard with IC_{50} value of 0.85±0.0001 µmol, probably due to the *S*-substitution of 2-chlorobenzyl and 4-bromobenzyl groups respectively in these molecules. However, only some compounds (Table-1) showed weak inhibition against lipoxygenase enzyme but all other compounds remained inactive. DPPH is a stable free radical at room temperature. DPPH radical is scavenged by antioxidants through the donation of a proton and form reduced DPPH. The colour changes from violet to yellow after reduction of DPPH, and it can be quantified by decrease of absorbance at wavelength 517 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition [21]. The colour change from violet to yellow and fall in absorbance of the stable radical DPPH was measured for three different concentrations of samples, and the results are shown in Table 1. These results showed that only two compound 4 and 6a showed IC50 values at 75.21±0.11 and 182.71±0.61 respectively against standard quercetin of DPPH. The IC_{50} value for each sample was calculated from the curves plotted. IC_{50} is the concentration of fraction causing 50 percent inhibition of absorbance and lower its value means greater antioxidant activity of the fraction.

CONCLUSION

The proposed structure of the synthesized compound is well supported by spectroscopic data. From the enzyme inhibition data (Table 1), it may be concluded that the compounds have moderate to talented activity against acetylcholinesterase, butyrylcholinesterase and lipoxygenase enzymes as it was evident from their IC_{50} values, relative to the standard used. Only two compounds showed scavenging activity against DPPH but all others stayed inactive. Hence, on the basis of aforesaid results, these synthesized derivatives provide an overall indispensable basis to introduce new drug candidates for the treatment of Alzheimer's disease and other associated diseases. These entrants can also be helpful for the treatment of a variety of disorders such as bronchial asthma, inflammation, cancer, and autoimmune diseases.

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