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Synthesis, Characterization and Biological activity of 5-(2-aminophenyl)-1,3,4-oxadiazole-2(3H)-thione

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Abstract
In the present paper 5-(2-aminophenyl)-1,3,4-oxadiazole-2(3H)-thione was synthesied synthesized from 2-amino benzoic acid (1b) (10 g, 0.073 mol) as the starting material esterified to methyl 2-amino benzoate (2b) in presence of conc. Sulphuric acid, further methyl benzoate refluxed for 5 hrs with hydrazine hydrate in ethanol to get 2-aminobenzohydrazide (3b) (1.51 g, 0.01 mol) further refluxed with carbon disulphide (10 mL) in alkaline medium to get 5-(2-aminophenyl)-1,3,4-oxadiazole-2(3H)-thione (4b). The synthesized 5-(2-aminophenyl)-1,3,4-oxadiazole-2(3H)-thione was further evaluated and are reported.

Key-words: *Oxadiazoles, Derivatives, Synthesis*

Introduction

A variety of heteroaromatic/aromatic ring-containing compounds were produced in order to assess their potential as AChE/BChE inhibitors as well as their antioxidant capabilities. Examples of these compounds include imidazole, acridine, cinnamide-dibenzylamine, p-aminobenzoic acid, triazine, and derivatives of benzoxazole. [1-3] Oxadiazoles are a significant class of heterocyclic compounds with a diverse range of biological activity. Substituted 1,3,4-oxadiazoles have been shown to exhibit antibacterial, antimycobacterial, antifungal, anti-inflammatory, analgesic, anticonvulsant, and anticancer effects [4]. Oxadiazole is thought to be derived from furan by replacing two methane (-CH=) groups with two pyridine type nitrogen atoms (-N=). Several methods have been documented in the literature for synthesising 1,3,4-oxadiazoles. [5]

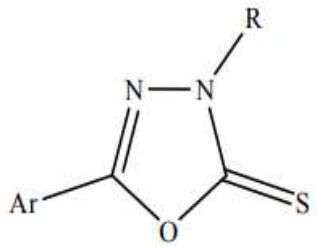


Fig. 1: Nucleus of 5-substituted-1,3,4-oxadiazole-2-thione

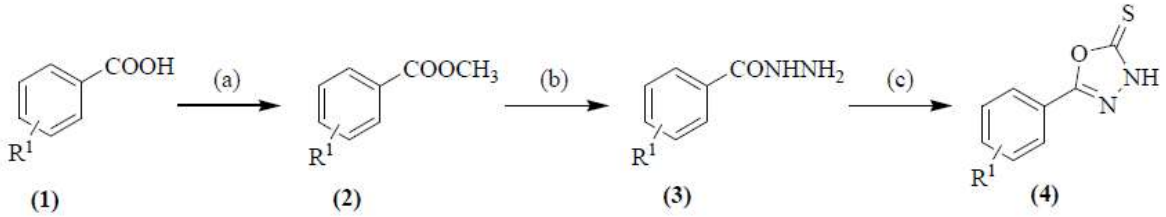
Experimental Methods

Chemicals, Techniques, and Instruments Used

The chemicals, solvents employed for synthetic work were of BDH, Hi-media, E-Merck, Loba, and Laboratory grade. The solvents further purified by established methods. Few of chemicals used in synthesis obtained from Sigma, Aldrich USA. All the residues dried in vacuum desiccators. The percentage yields are base upon the products obtained after purification through crystallization. The solvent used for crystallization has been mention within brackets after melting point (mp).The melting points of the compounds were determined in open capillaries using Thermonik Precision Melting point cum Boiling point apparatus (C-PMB-2, Mumbai, India). The melting points reported here in are in the Celsius scale and are uncorrected. Precoated silica gel-G plate activated at 110° C for 30 min. used for thin layer chromatography and the spots developed in iodine chamber. Though different solvent systems were employed, R_f values are reported for better comparable solvent systems, which are mentioned in the preceding text. The ultraviolet spectra measured in variable solvents (HPLC grade) on Shimadzu 1601 Spectrophotometer. IRspectra of compounds recorded using KBr pellets on FTIR-8400s, Shimadzu make, at Sharad Pawar College of Pharmacy, Wanadongri, Hingna Road, Nagpur. ^{1}H-NMR[11] spectra ($CDCl_3$, DMSO d_6) were recorded on Varian EM 390 Spectrophotometer (chemical shift in ppm), at Department of Chemistry, Pune University, Pune. The chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) in $CDCl_3$/DMSO solution. Signal multiplicities are present by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet).^{13}C-NMR spectra ($CDCl_3$, DMSO d_6) were recorded on Bruker Avance II 400 NMR Spectrometer (chemical shift in ppm), at Sophisticated Analytical Instrument Facility (SAIF), Panjab University, Chandigarh. The chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) in $CDCl_3$/DMSO solution. [4-10].LC-MS spectra were also recorded.

Synthesis

5-Substituted-1,3,4-oxadiazole-2-thiones **(4)** will be prepared according to the method described in literature. In the initial step substituted benzoic acid **(1)** will be esterified to its benzoate **(2)** in the presence of concentrated sulphuric acid. Subsequently the ester **(2)** will be refluxed with excess of hydrazine hydrate in ethanol to afford the hydrazide **(3)**. The hydrazide **(3)** will be annulated to respective 5-substituted-1,3,4-oxadiazole-2-thione **(4)** by reacting with carbon disulfide (CS_2) in ethanolic alkaline medium (**Scheme I**). **Scheme I:** Reagents and reaction conditions: (a) CH_3OH, H_2SO_4, reflux; (b) C_2H_5OH, $NH_2NH_2.H_2O$, reflux; (c) C_2H_5OH, KOH**,** CS_2, reflux. [6-7]



Compd. No.				R^1
(1a)	(2a)	(3a)	(4a)	-H
(1b)	(2b)	(3b)	(4b)	2-NH_2
(1c)	(2c)	(3c)	(4c)	4-NH_2
(1d)	(2d)	(3d)	(4d)	2-OH

(1e)	**(2e)**	**(3e)**	**(4e)**	4-NO_2
(1f)	**(2f)**	**(3f)**	**(4f)**	3-NO_2
(1g)	**(2g)**	**(3g)**	**(4g)**	4-Cl

5-substituted-1,3,4-oxadiazole-2-thione (4) [Scheme I]

General procedure:

In the initial step substituted benzoic acid (**1a-g)** will be esterified to its substituted methylbenzoate **(2a-g)** in the presence of concentrated sulphuric acid. To 10g (0.082 mol) of benzoic acid add 0.62 mol of methanol in a 100 mL round-bottomed flask. Cautiously, add 3 mL concentrated H_2S0_4 down the side of the flask. After gently swirling the contents of the flask, attach a reflux condenser and reflux the mixture for about 60 min. Allow the solution to cool. Add 50 mL of water to a separatory funnel, and then add the contents of the flask. Wash sequentially with 25 mL water and 25 mL 0.6M sodium bicarbonate. After shaking and venting the funnel, remove the sodium bicarbonate layer and test to see if it is basic. Then wash the organic layer with NaCl (salt) solution, separate the organic layer and dry with anhydrous magnesium sulfate. The aqueous layer from the sodium bicarbonate wash should be acidified with concentrated HCl. Weigh the sample of methyl benzoate and determine the yield.

Take 0.01 mol of substituted methylbenzoate **(2a-g**) add 0.02 mol of hydrazine hydrate and add 30 mL of ethanol and reflux for 6 hrs. The excess hydrazine and methanol were evaporated to give the crude product which was recrystallized from methanol to yield pure substituted benzohydrazide **(3a–g)**.

A mixture of 0.01 mol of substituted benzoylhydrazide (Rauf et al., 2007) **(3a–g)**, 0.01 mol of potassium hydroxide and 10 mL of carbon disulfide was refluxed (80–90 C) in 50 mL ethanol for 8 h. The reaction mixture was concentrated on water bath, then cooled to room temperature, acidified with dil. HCl at 0°C and the solid product was separated out. After that the product was filtered and washed with cold water. The solid products **(4a-g)** were then air dried. Further, the products **(4a-g)** were purified by silica gel column chromatography with petroleum ether: diethyl ether as eluent. The products were identified by spectral data. By adopting the similar procedure the compounds (**4a-g**) was prepared.

Biological Activity

***In-vivo* Studies**

Animals: *In-vivo* studies were performed on adult male Swiss albino mice (25-30 g), which were purchased from Central Animal Breeding House, Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India. The animals were kept in groups of six per polyacrylic cage and were given semisynthetic balanced diet and water *ad libitum*. The animals were kept at a temperature of 25 ± 2 °C with a relative humidity of 55 ± 10% under 12 h light/dark cycles. Different animals were used for each behavioral investigation. The study protocols were approved by the institutional animal ethics committee.

Acute oral toxicity study: The acute oral toxicity of synthesized compounds, were evaluated on healthy Swiss albino mice (25-30g) as per the OECD-423, 2001 guidelines. The test compound was administered at various doses up to 2000 mg/kg p.o., and the animals were observed at 30 min, 2 h, 4 h and 24 h to determine changes in their autonomic and behavioral responses. The animals were also observed for tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma for 14 days. [8]

Antioxidant activity [DPPH radical scavenging activity]

The nitrogen centered stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) has often been employd to characterized antioxidant property. It is reduced reversibaly and the odd electron in the DPPH free

radical gives purple colour having strong absorption maxima at 517 nm. This property is suitable for spectrophotometer studies. A radical scavenging antioxidant reacts with DPPH stable free radical and converts it into 1,1-diphenyl-2-picrylhydrazine. The resulting stoichometric decolorization is in proportion with respect to the number of electrons captured. The absorbance change produced in this reaction has been use to calculate antioxidant properties of test compounds [9]. Compounds were evaluated for their *in vitro* antioxidant activity by DPPH free radical scavenging assay method. To determine the free radical scavenging activity, a method based on the reduction of a methanolic solution of the coloured DPPH radical was used. To a set of test tubes containing 3 mL of methanol, 50 µL of DPPH reagent (2 mg/mL) added. The initial absorbance measured. To these test tubes, methanolic solution of different test solutions (1 mg/mL) were added (10-50 µL). Ascorbic acid (0.5 mg/mL) also added in the concentration of 10, 20, 30, 40, 50 and 100 µL. Absorbance recorded at 516 nm after 20 minutes of addition. All experiments repeated for three times and average is calculated. The percentage reduction in absorbance calculated from the initial and final absorbance of each solution. Percentage scavenging of DPPH radical calculated using the formula

% Scavenging of DPPH = [(Control-Test)/Control] × 100

Antimicrobial Activity

The synthesized compounds were subjected for *in vitro* antimicrobial activity by agar diffusion method using Gram Positive (*S. aureus ATCC 25923* and *B. substilis ATCC 6051*), Gram negative (*E. coli ATCC25922* and *K. aerogens ATCC27853*) bacteria strain and two strains of fungi i.e. of *C. albicans* and *A. niger.* [10-11]

Media, Stock Solution and Control Parameters

Composition of media:

A) Antibiotic Assay Medium (for subculturing) (for antibacterial activity)

Peptone	:	6.0 g
Pancreatic digest of casein	:	4.0 g
Yeast extract	:	3.0 g
Beef extract	:	1.5 g
Dextrose	:	1.0 g
Agar	:	15 g
Distilled water	:	up to 1000 mL (pH 6.6)

B) Sabouraud Dextrose Agar Medium (for subculturing) (for antifungal activity)

Dextrose	:	40 g
Mixture of equal parts of peptic digest of animal tissue and pancreatic digest of casein	:	10 g
Agar	:	15 g
Distilled water	:	up to 1000 mL (pH5.6 ± 0.2)

The medium sterilized by autoclaving at 15 lb pressure for 30 minutes. One loopful of the stock culture inoculated at 10 mL of agar slant in previously sterilized test tubes, and incubated at 37° for 24 h and 20° for 48 h to 7 days, for bacteria and fungi respectively. About 3 mL of distilled water was added to the test tube and by shaking for few minutes, a suspension of the culture was obtained.

Stock solution:

The test compounds (100 mg) were dissolved in methanol (10 mL), and volume was make upto 100 mL with sterilized water to produced a concentration of 100 µg/mL. Similarly, the dilutions were prepared for standard drugs i.e., norfloxacin and clotrimazole.

Antimicrobial Protocol

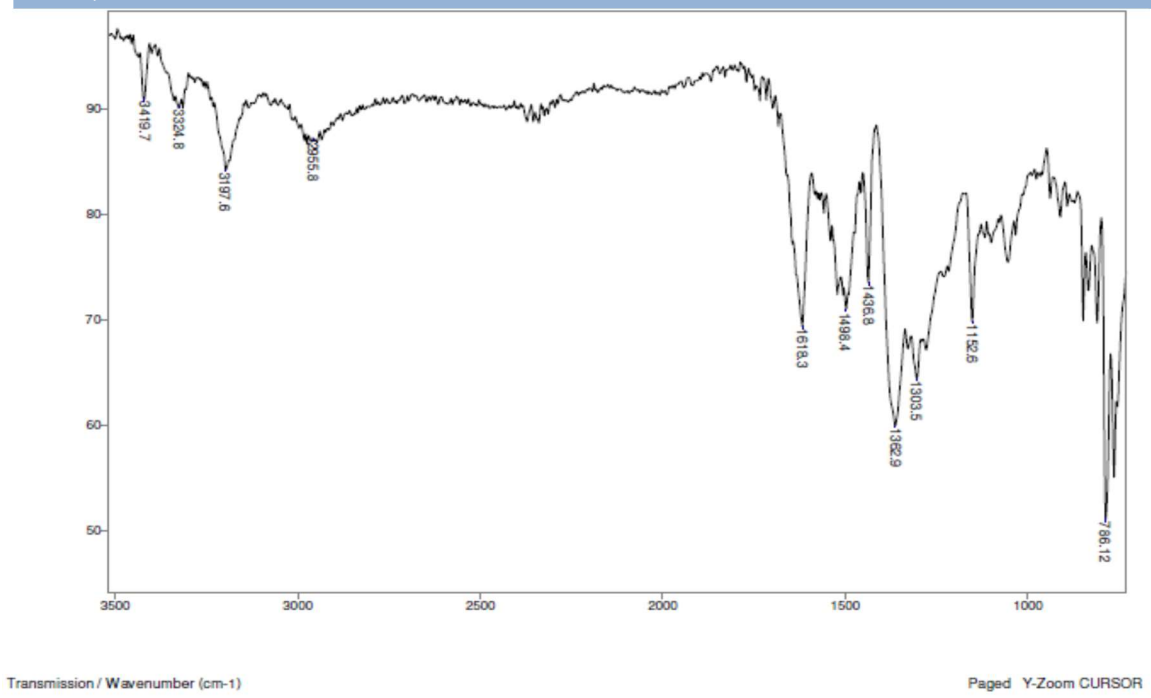
The preliminary antibacterial activity of synthesized compounds was studied against two different strains of Gram-negative *Escheria coli* and *Klebsilla aerogens* and Gram-positive *Staphyllococci aureus*, *Bacillus substilis* bacteria by agar plate method. Antifungal activity of compounds was estimated against *Candida albicans*, *Asperigillus niger* by agar plate method. Norfloxacin and clotrimazole used as standards, for antibacterial and antifungal activity respectively. Screening of antimicrobial activity carried out using the cup plate method. This method depends on the diffusion of drug from cup through the solidified agar layer of a petridish to an extent, such that growth of the inoculated microorganism is prevented entirely in a circular area "zone" around the cup containing the solution of the compound under test.

Results and Conclusion

5-(2-aminophenyl)-1,3,4-oxadiazole-2(3H)-thione (4b)

Following general procedure as described above (**4b**) was synthesized from 2-amino benzoic acid (**1b**) (10 g, 0.073 mol) as the starting material esterified to methyl 2-amino benzoate (**2b**) in presence of conc. Sulphuric acid, further methyl benzoate refluxed for 5 hrs with hydrazine hydrate in ethanol to get 2-aminobenzohydrazide (**3b**) (1.51 g, 0.01 mol) further refluxed with carbon disulphide (10 mL) in alkaline medium to get 5-(2-aminophenyl)-1,3,4-oxadiazole-2(3H)-thione (**4b**).

Yield: 9.89 g (70.19%), mp. 189-191° (ethanol), R_f 0.57 (chloroform: ethanol, 3:1), λ_{max} (methanol) (lgε)/nm 285. FT-IR: (KBr, cm^{-1}) 3642.07-3593.89 (Thioamide N-H stretching), 3474.82-3372.06 (Ar N-H stretching), 3037.4 (Ar C-H stretching), 1618.5 (Ar C-C stretching), 1361.84-1279.7 (oxadiazole N-H stretching), 1292-1366.12 (oxadiazole C-N stretching), 847.83-767.46 (oxadiazole C(=S)-N stretching), 713.38-665.82 (oxadiazole C-O stretching) **(Graph 4)**. *m/z* (GC-MS): 193.03 (100%, Base peak, M^+). Found C 48.69, H 3.82, N 21.58, O 8.24, S 16.75 %. $C_8H_7N_3OS$ (193.22) requires C 49.73, H 3.65, N 21.75, O 8.28, S 16.59 %.



Graph 4

In vivo Study

Acute oral toxicity study: Acute oral toxicity was determined for the compounds using OECD guidelines 423. The results were given in table 1. The results obtained indicate that synthesized compounds are safe and non-toxic till dose of 2000 mg/kg bw. Hence ED 50 will be considered as 200 mg/kg bw.

Table 5.1: Determination of LD_{50} and ED_{50} of synthesized compounds

Compounds				**Mortality at dose 2000 mg/kg bw**
1(a)	2(a)	3(a)	4(a)	0
1(b)	2(b)	3(b)	4(b)	0
1(c)	2(c)	3(c)	4(c)	0
1(d)	2(d)	3(d)	4(d)	0
1(e)	2(e)	3(e)	4(e)	0
1(f)	2(f)	3(f)	4(f)	0
1(g)	2(g)	3(g)	4(g)	0

Anti-oxidant Activity

Anti-oxidant activities of synthesized compounds were determined using DPPH methods. Results presented in table 2 indicate that % inhibition of compounds.

Table 5.3: % Inhibition of synthesized compounds using DPPH method

S/No.	**Synthesized Compounds**	**% Inhibition**
1.	**Ascorbic Acid**	**78.63**
2.	1(a)	32.19
3.	1(b)	30.46
4	1(c)	28.48

5	1(d)	32.13
6	1(e)	32.98
7	1(f)	39.75
8	1(g)	41.54
9	2(a)	34.11
10	2(b)	40.98
11	2(c)	42.32
12	2(d)	44.01
13	2(e)	35.52
14	2(f)	32.11
15	2(g)	41.32
16	3(a)	43.50
17	3(b)	44.00
18	3(c)	39.99
19	3(d)	35.54
20	3(e)	37.02
21	3(f)	34.44
22	3(g)	33.15
23	4(a)	34.05
24	4(b)	38.12
25	4(c)	34.19
26	4(d)	42.09
27	4(e)	41.12
28	4(f)	34.99
29	4(g)	44.09

Anti-microbial Activity

Anti-microbial activity of synthesized compounds was determined and results were presented in table 3. The results obtained showed satisfactory anti-microbial activity.

Table 5.3: Antimicrobial activity-sensitivity testing of compounds

Compounds	**Zone of Inhibition (mm)**					
	Anti-bacterial activity				**Anti-fungal activity**	
	S. aureus	***B. substilis***	***E. coli***	***K. aerogens***	***C. albicans***	***A. niger.***
Norfloxacin	**21**	**19**	**22**	**18**	**-**	**-**
Clotrimazole	**-**	**-**	**-**	**-**	**20**	**22**
1(a)	8	11	-	8	-	10
1(b)	7	6	11	9	17	11
1(c)	5	2	8	6	5	9
1(d)	8	9	7	9	11	10
1(e)	10	11	9	-	9	-
1(f)	11	-	11	-	-	-
1(g)	8	10	-	-		8
2(a)	11	8	9	13	8	-
2(b)	8	10	11	5	7	11
2(c)	10	-	6	11	8	9
2(d)	9	17	-	-	9	17
2(e)	6	5	9	17	6	5
2(f)	9	11	6	5	9	11
2(g)	9	11	9	11	11	-
3(a)	9	17	9	17	-	-
3(b)	6	5	6	5	9	17
3(c)	9	11	9	11	6	5
3(d)	-	-	-	-	9	11
3(e)	9	13	-	-	-	-
3(f)	11	5	-	-	-	-
3(g)	6	11	-	-	-	-
4(a)	10	11	5	7	11	-
4(b)	-	8	-		-	-
4(c)	9	-	11	5	7	11
4(d)	-	-	-	-	-	-
4(e)	10	11	5	7	11	-
4(f)	8	-	-	9	13	-
4(g)	9	17	-	11	5	9

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