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Synthesis, characterization and anticancer activity of 1,2,4-Triazolo [3,4-b]-1,3,4-thiadiazoles on Hep G2 cell lines

Dhanya Sunil[1], Arun M Isloor[2]* and Prakash Shetty[3]

[1]Department of Chemistry, Manipal Institute of Technology, Manipal University,India
*[*2]Department of Chemistry, National Institute of Technology, Surathkal, Mangalore, India*
[3]Department of Printing and Media Engineering, Manipal Institute of Technology, Manipal University, India

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Abstract

A series of 3,6-disubstituted 1,2,4–triazolo[3,4-b]-1,3,4-thiadiazoles were synthesized from 3-substituted-4-amino-5-mercapto-1,2,4-triazoles and 3-substituted 4-carboxypyrazoles. Elemental analyses, IR, ^{1}H NMR and mass spectral data confirmed the structures of all newly synthesized compounds. Most of the newly synthesized compounds were screened for their anticancer activity in hepatic cell lines. Many of the compounds were found to be potent. The perturbations brought by the substituent can affect various parameters of the molecule like its electron density, its steric environment, its bioavailability etc. The thiadiazole with naphthyloxymethyl and fluorophenyl groups as substituents showed excellent antiproliferative effect.

Keywords*:* 1,2,4-triazoles; 4-carboxypyrazoles; 1,2,4 –triazolo [3,4-b]-1,3,4-thiadiazoles and anticancer activity.

Introduction

Recently it was reported that, 1,2,4–triazoles and 1,3,4-thiadiazoles possess variety of broad spectrum biological activities. In last few decades, triazoles have received much significant attention in the field of medicinal chemistry because of their diversified biological properties like antibacterial [1-3], antifungal [4], antiviral [5], anti-inflammatory [6,7], analgesic [8], antimicrobial [9,10], anti-hypertensive [11], antitubular [12] and anticancer [13-15] properties. The amino and mercapto groups of 1,2,4-triazoles serve as readily accessible nucleophilic centers for the preparation of N- bridged heterocycles. Further, current literature indicates 1,2-pyrazole derivatives to possess various biological activities [16].

1,3,4-thiadiazoles exhibit wide spectrum of biological activities, possibly due to presence of toxophoric >N-C-S- moiety [17]. They find applications as antibacterial [17, 18], antimicrobial [9, 10] and anti-inflammatory agents [18]. A triazolo thiadiazole system may be viewed as a cyclic analogue of two very important components thiosemicarbazide and biguanide which often display diverse biological activities. The 1,2,4-triazolo[3,4-b]-1,3,4-thiadiazole derivatives obtained by reacting the bio-labile 1,2,4- triazole and 1,2-pyrazole rings together are reported to possess antibacterial [17,18], antifungal [17,18], anti-inflammatory [17,18], antioxidant [17], analgesic [18], and antimicrobial [9,10], activities.

The incorporation of halogen atoms and aryloxymethyl substituent into the heterocyclic ring systems enhances the biological activities to a great extent. Recently some bis triazolo-thiadiazoles endowed with excellent anticancer activities have been reported. The choice of the optimal substituent allows noticeable gains in potency, compared to parent molecules. Prompted by these observations and in continuation to our previous work [19], we report herein the synthesis of novel class of 1,2,4–triazolo [3,4-b]-1,3,4-thiadiazoles and their anticancer screening.

Results and Discussion

The characterization data of triazolo-thiadiazoles are shown in Tables 1 and 2. Formation of triazolo-thiadiazoles was confirmed by recording their IR, ^{1}H-NMR and mass spectra. The progress of the reaction was monitored by TLC. The purity of the compounds was checked using gas chromatography using Agilent 6320 Ion Trap. IR spectrum of triazolo-thiadiazole **4a** showed an absorption band at 1240 cm^{-1} indicating the presence of >N-N=C in the molecule. An absorption band at 1033 cm^{-1} due to the presence of -C-S- bond was also observed.

The ^{1}H- NMR spectrum of **4a** showed a singlet at δ 5.2 corresponding to OCH_2 protons. The peak at δ 7.1 was due to N-H protons of pyrazole. C-H protons of pyrazole ring formed a peak at δ 6.9. The peaks at δ7.62 – 8.0 were due to protons of aromatic rings.

The mass spectrum of **4a** showed molecular ion peak at *m/z* 408 which was in agreement with its molecular formula $C_{19}H_{13}ClN_6OS$. The peak that appeared at *m/z* 205 as base peak, accounted for the formation of 5-(phenoxy methyl)-1,2,4-triazole-3-thiol cation. Peak at *m/z* 77 was due to the phenyl group. M^+ of 6-[3-(4-chlorophenyl)-1H-pyrazol-4-yl]-3-methyl [1,2,4]triazolo[3,4-b]]1,3,4]thiadiazole showed a peak at *m/z* 316.

Results of anticancer studies of triazolo-thiadiazoles are given in Table 3 (%survival in four different concentrations and IC_{50}).The replacement in an active molecule, of a hydrogen atom by substituents like alkyl, halogens etc deeply modified the potency of the compounds. Compounds **4f** and **4m** were found to show excellent antiproliferative activity even at very low concentration of 1 µg/ml with IC_{50} values of 0.4 µg/ml and 0.1 µg/ml. **4f** had o-cresyloxymethyl and p-methoxyphenyl groups as substituents. The increase in lipophilicity can be attributed to the methyl groups which can drastically modify the bioavailability of the compound. **4m** had

Table-1 Characterization of 3-substituted-6-(3-substituted-1H-pyrazol-4-yl)[1,2,4] triazolo[3,4-b][1,3,4]thiadiazole

Com No.	R	R_1	M.P (^{o} C) and Yield (%)	Molecular formula	Crystal nature	Analysis % Found (Calculated)		
						C	H	N
4a	Phenoxymethyl	p-Chlorophenyl	155 73	$C_{19}H_{13}ClN_6OS$	Pale brown crystals	55.80 (55.81)	3.01 (3.18)	20.39 20.56)
4b	Phenoxymethyl	p-Fluorophenyl	198 82	$C_{19}H_{13}FN_6OS$	Mustard wool	58.08 (58.16)	3.29 (3.32)	21.40 21.42)
4c	Phenoxymethyl	p-Methoxyphenyl	130 69	$C_{20}H_{16}N_6O_2S$	Brown crystals	59.32 (59.41)	3.84 (3.96)	20.78 20.79)
4d	o-Cresyloxymethyl	p-Chlorophenyl	152 73	$C_{20}H_{15}ClN_6OS$	Pale brown flakes	56.79 (56.80)	3.49 (3.55)	19.80 19.88)
4e	o-Cresyloxymethyl	p-Fluorophenyl	182 69	$C_{20}H_{15}FN_6OS$	Cream crystals	59.08 (59.11)	3.61 (3.69)	20.59 (20.69)
4f	o-Cresyloxymethyl	p-Methoxyphenyl	108 61	$C_{21}H_{18}N_6O_2S$	Pale brown needles	60.17 (60.29)	4.21 (4.31)	20.12 (20.10)
4g	p-Cresyloxymethyl	p-Chlorophenyl	178 81	$C_{20}H_{15}ClN_6OS$	Mustard flakes	56.69 (56.80)	3.59 (3.55)	19.80 (19.88)
4h	p-Cresyloxymethyl	p-Fluorophenyl	181 75	$C_{20}H_{15}FN_6OS$	Cream flakes	59.15 (59.11)	3.72 (3.69)	20.60 (20.69)
4i	p-Cresyloxymethyl	p-Methoxyphenyl	90 62	$C_{21}H_{18}N_6O_2S$	Brown needles	60.30 (60.29)	4.33 (4.31)	20.14 (20.10)
4j	o-Chlorophenoxymethyl	p-Chlorophenyl	98 68	$C_{19}H_{12}Cl_2N_6OS$	Brown needles	51.39 (51.47)	2.74 (2.71)	18.89 (18.96)
4k	o-Chlorophenoxymethyl	p-Fluorophenyl	118 72	$C_{19}H_{12}ClFN_6OS$	Brown flakes	53.39 (53.46)	2.89 (2.81)	19.61 (19.70)
4l	o-Chlorophenoxymethyl	p-Methoxyphenyl	168 67	$C_{20}H_{15}ClN_6O_2S$	Brown flakes	54.73 (54.80)	3.42 (3.50)	19.16 (19.28)
4m	2-Naphthyloxymethyl	p-Fluorophenyl	158 85	$C_{23}H_{15}FN_6OS$	Cream needles	62.44 (62.40)	3.39 (3.37)	19.01 (19.12)
4n	2-Naphthyloxymethyl	p-Methoxyphenyl	129 71	$C_{24}H_{18}N_6O_2S$	Brown crystals	63.38 (63.44)	4.03 (3.96)	18.59 (18.50)

Table-2 Spectral characterization of 3-substituted-6-(3-substituted-1H-pyrazol-4-yl)[1,2,4] triazolo[3,4-b][1,3,4]thiadiazole

Com No.	IR (KBr) υ (cm^{-1})	^{1}H-NMR δ (ppm) ($CDCl_3$, 400MHz)	MS *m/z*
4[a]	1600 (C=N), 3081,3032 (aromatic C-H str.), 1590,1540,1490,1450 (C=C ring str.)1250 (asymmetric C-O-C str.),1045 (symmetric C-O-C str.)1240 (N-N=C), 1033 (C-S)	7.1 (1H, N-H of pyrazole), 5.2(s, 2H , O-CH_2), 6.9 (s, 1H, C-H of pyrazole), 7.62 – 8.0 (H in benzene ring)	408 {M^+},205 {M^+ of 5-(phenoxy methyl)-1,2,4-triazole-3-thiol}, 203 { M^+ of 3-(4-chlorophenyl)-4-vinyl-1H pyrazole}
4b	1603 (C=N), 3085,3031 (aromatic C-H str.), 1593,1545,1497,1454 (C=C ring str.)1254 (asymmetric C-O-C str.),1049 (symmetric C-O-C str.)1243 (N-N=C), 1035 (C-S)	7.1 (1H, N-H of pyrazole), 5.21(s, 2H , O-CH_2), 6.91 (s, 1H, C-H of pyrazole), 7.16 – 7.75 (H in benzene ring)	392 {M^+}, 296 { M^+ of 3-(phenoxy methyl)-6-(1H-pyrazol-4yl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole}
4c	1605 (C=N), 3080, 3039 (aromatic C-H str.), 1590, 1540, 1490, 1450 (C=C ring str.)1250 (asymmetric C-O-C),1045 (symmetric C-O-C str.)1240 (N-N=C),1033 (C-S)	7.1 (1H, N-H of pyrazole), 5.2(s, 2H , O-CH_2), 3.85 (3H, OCH_3), 6.9 (s, 1H, C-H of pyrazole), 7.14 – 7.95 (H in benzene ring)	404 {M^+}, 205 {M^+ of 5-(phenoxy methyl)-1,2,4-triazole-3-thiol}, 231 { M^+ of 3-(phenoxy methyl)[1,2,4]triazolo[3,4-b]thiadiazole}
4d	1603 (C=N), 3082, 3037 (aromatic C-H str.), 1590, 1540, 1490, 1450 (C=C ring str.)1250 (asymmetric C-O-C), 1045 (symmetric C-O-C str.)1240 (N-N=C),1033 (C-S)	7.1 (1H, N-H of pyrazole) 5.2 (s, 2H , O-CH_2) 2.48 (3H, CH_3) 6.9 (s, 1H, C-H of pyrazole) 7.19 - 8.0(H in benzene ring)	422.5 {M^+}, 219 {M^+ of 5-[(2-methyl phenoxy) methyl]-1,2,4- triazole-3-thiol}, 316 { M^+ of 6-[3-(4-chlorophenyl)-1H-pyrazol-4-yl]-3-methyl [1,2,4]triazolo[3,4-b]]1,3,4]thiadiazole}
4e	1600 (C=N), 3080, 3037 (aromatic C-H str.), 1590, 1540, 1490, 1450 (C=C ring str.)1250 (asymmetric C-O-C),1045 (symmetric C-O-C str.)1240 (N-N=C),1033 (C-S)	7.1 (1H, N-H of pyrazole) 5.22 (s, 2H , O-CH_2) 2.48 (3H, CH_3) 6.9 (s, 1H, C-H of pyrazole) 7.19 – 7.77(H in benzene ring)	406 {M^+}, 189 {M^+ of 3-(4-fluorophenyl)-4-vinyl-1H pyrazole}
4g	1604 (C=N), 3083, 3039 (aromatic C-H str.), 1590,1520, 1490, 1460 (C=C ring str.), 1243(asymmetric C-O-C), 1045 (symmetric C-O-C str.)1240 (N-N=C), 1033 (C-S)	7.1 (1H, N-H of pyrazole) 5.21 (s, 2H , O-CH_2) 2.25 (3H, CH_3) 6.9 (s, 1H, C-H of pyrazole) 7.11 – 8.00(H in benzene ring)	422.5 {M^+}, 301 { M^+ of 6-[3-4-chlorophenyl)-1H-pyrazol-4-yl][1,2,4]triazolo[3,4-b][1,3,4]thiadiazole}
4h	1605 (C=N), 3080, 3038 (aromatic C-H str.) 1590,1520, 1490, 1460 (C=C ring str.) 1248(asymmetric C-O-C), 1045 (symmetric C-O-C str.)1233(N-N=C), 1031 (C-S)	7.10 (1H, N-H of pyrazole) 5.2 (s, 2H , O-CH_2) 2.3 (3H, CH_3) 6.92 (s, 1H, C-H of pyrazole) 7.19 – 7.77 (H in benzene ring)	406 {M^+}, 190 {M^+ of 6-(1H-pyrazol-4-yl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole}
4m	1610 (C=N), 1590,1508, 1492, 1460 (C=C ring str.) 1025 (C-F str.) 3200 (N-H str.) 1580 (N-H bend)	7.10 (1H, N-H of pyrazole) 6.92 (s, 1H, C-H of pyrazole) 7.22 – 7.87 (H in naphthalene ring) 5.2 (s, 2H , O-CH_2)	442 {M^+}, 299 { M^+ of 6-[3-(4-fluorophenyl)-1*H*-pyrazol-4-yl]-3-methyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole

Table-3 Anticancer screening data of 3-substituted-6-(3-substituted-1H-pyrazol-4-yl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (% survival in four different concentrations)

Com No	control	S.D	Vehicle control	S.D.	0.01 µg/ml	S.D.	0.1 µg/ml	S.D.	1µg/ml	S.D.	10 µg/ml	S.D.	IC_{50} (µg/ml)
4a	100	2.4	94.2	2.7	81.7	3.2	73.0	3.2	38.1	2.0	10.1	0.2	0.7
4b	100	2.4	94.2	2.7	90.0	3.2	63.1	0.8	47.3	2.2	33.3	2.7	0.8
4c	100	2.4	94.2	2.7	90.1	2.9	83.2	3.0	79.5	2.9	32.9	6.1	6.7
4d	100	8.3	95.9	2.1	90.3	5.1	80.5	5.3	69.9	5.1	33.7	5.2	5.8
4e	100	2.0	93.7	0.9	78.7	5.0	71.5	11.	60.6	11.8	1.6	1.2	2.6
4f	100	2.0	93.7	0.9	73.1	6.1	60.5	2.7	31.1	4.7	8.3	1.5	0.4
4g	100	8.3	95.9	2.1	94.8	0.3	90.5	3.5	80.2	5.7	18.8	2.8	5.4
4h	100	1.93	95.3	4.7	97.5	4.0	96.3	3.9	84.6	1.3	45.7	2.1	8.9
4j	100	8.3	95.9	2.1	82.7	4.9	65.9	15.7	20.4	10.2	16.3	2.9	0.4
4m	100	8.3	95.9	2.1	75.6	1.8	33.0	8.1	17.0	7.3	7.2	1.3	0.1

S.D. – standard deviation

naphthyloxymethyl and fluorophenyl groups as substituents. Presence of fluoro-substituents and aromatic naphthalene rings were found to enhance the anti-neoplastic character of the respective compounds. The difference in electro negativity between fluorine and carbon created a large dipole moment which contributed to the molecule's ability to be engaged in intermolecular interactions.

Triazolo-thiadiazoles **4a**, **4c**, **4d**, **4e** and **4g** were found to show significant activity in 10µg/ml concentration. All the above mentioned compounds had halogen and aryloxy substituents, which accounted for their significant anti-proliferative activity.

Materials and Methods

All the chemicals used in the present study were from Sigma-Aldrich, USA. 3-substituted 4-amino-5-mercapto-1,2,4-triazoles **1** are versatile synthones for the construction of various biologically active heterocycles. 3-Substituted 4-amino-5-mercapto-1,2,4-triazoles **1** were synthesized as per literature method [20]. Substituted phenols on reaction with ethyl chloroacetate & potassium carbonate in dry acetone medium yielded the ester which when refluxed with hydrazine hydrate gave the corresponding hydrazide. Further these hydrazides were stirred with potassium hydroxide & carbon disulphide in absolute ethanol medium to obtain potassium salts. The salts obtained were refluxed with hydrazine hydrate to yield the substituted 1,2,4-triazoles. 3-Substituted pyrazole aldehydes were prepared as reported in literature [21]. Ketones were treated with semicarbazide hydrochloride in presence of sodium acetate to yield corresponding semicarbazones. Further on Vilsmeier Haack reaction of semicarbazones yielded 3-substituted pyrazole aldehyde.

These aldehydes were converted into pyrazole acids by oxidation, using potassium permanganate in pyridine medium [22]. Subsequently 3-substituted 4-amino-5-mercapto-1,2,4-triazoles **1** and

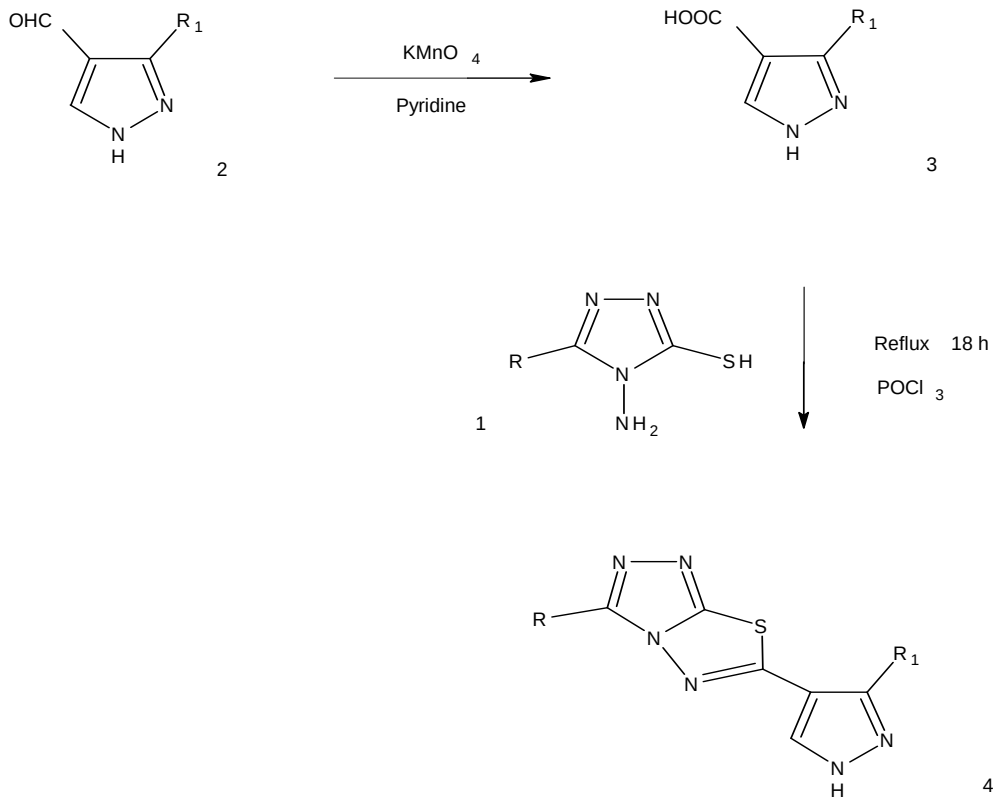
3-substituted pyrazole acids **3** were refluxed for 18h in phosphorous oxychloride which resulted in cyclized title compounds.

Experimental

*3-(4-substitutedphenyl)-1H-pyrazole-4-carboxylic acid **3** (Scheme-1)*

0.01 moles of pyrazole aldehyde was dissolved in 10ml pyridine. 0.01 moles (1.58g) of potassium permanganate dissolved in 10ml water were added to the above suspension of pyrazole aldehyde at room temperature with stirring. The reaction mixture was stirred for 3h. Then 50ml of 1% sodium hydroxide solution was added and stirring was continued for 2 h at 50^0C. After cooling, the inorganic precipitate was filtered off and washed with water. The filtrate and the washings of precipitate were acidified with 6N hydrochloric acid to pH 4. The precipitate was filtered, washed with water, dried and recrystallized from glacial acetic acid. Three pyrazole acids with p-Chlorophenyl, p-fluorophenyl and p-methoxyphenyl as substituents were synthesized.

Scheme-1:Synthesisof3-substituted-6-(3-substituted-1H-pyrazol-4- yl)[1,2,4]triazolo [3,4-b][1,3,4]thiadiazole



*3-substituted-6-(3- substituted -1H-pyrazol-4-yl)[1,2,4]triazolo[3,4-b][1,3,4] thiadiazole **4a-4n** (Scheme-1)*

To a mixture of 0.01 moles of 3-substituted 4-amino-5-mercapto-1,2,4-triazole **1** and 0.01 moles of 3- substituted pyrazole acid **3**, 15ml of phosphorous oxychloride was added and contents were heated under reflux for 18h. Excess phosphorous oxychloride was then distilled off and the

residue was then poured onto crushed ice with vigorous stirring. The resulted solid was washed with cold water, 20% sodium hydrogen carbonate solution and then recrystallized from a mixture of ethanol and dioxane (1:1 mixture).

Characterization

All the compounds were recrystallized and purity was checked by gas chromatography. Thin layer chromatography was conducted on 0.25 mm silica gel plates to monitor the progress of the reaction and to check the purity of the compounds. A 1:1 mixture of ethyl acetate and petroleum ether solution was used as the eluent. Visualization was made by using iodine vapours. Melting points were determined by open capillary method. The IR spectra were recorded on a Schimadzu FTIR 8400S spectrophotometer using KBr pellets. ^{1}H-NMR spectra were recorded in $CDCl_3$/deuterated acetone on an AV500 NMR spectrometer using TMS as an internal standard. The mass spectra were recorded on a JEOL JMS 300 mass spectrometer operating at 70eV.

Pharmacology

Few of the selected newly synthesized triazolo-thiadiazoles were screened for their anticancer activities at Life Sciences Centre, Manipal University, India. Ten compounds were selected for MTT assay. The assay was conducted using HepG2 cells (Hepatic cell lines) growing in DMEM (Dulbecco's Modified Eagle's Medium) with 10% FBS (Fetal Bovine Serum) at exponential phase in tissue culture flasks. The cells were trypsinized and counted using standard procedures [23]. Cells were seeded at a density of 1 x 10^4 cells to each well in a 96 well plate. These were incubated for 24 hrs at 37^0C in carbon dioxide incubator prior to treatment with the newly synthesized triazolo -thiadiazoles. Subsequently, cells were treated with different concentrations of the compounds and incubated at 37^0C for 24hrs. Vehicles and their dilutions used to dissolve the compounds were also used as controls. After 24hrs, 5 milli moles of MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well and incubated at 37^0C for additional 4hrs to allow the formation of formazan crystals. Plates were centrifuged at 3000 rpm for 10 min, media was aspirated and 100µl of hydrochloric acid (0.4 N): isopropanol (1:24) was used to dissolve the formazan crystals. The optical density was measured at 570 nm in an ELISA plate reader.

The results were reported as percentage survival of the cells when compared to that of the untreated control cells. The standard deviation was calculated for each concentration of the compound and also for the control.

Conclusion

Triazolo thiadiazoles **4m** and **4f** showed remarkable anti-neoplastic activity at a very low concentration of 1 µg/ml. The presence of methyl, naphthyl and fluoro substituents enhanced their potency.

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