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Original article

Synthesis and biological evaluation of 3,6-disubstituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole derivatives as a novel class of potential anti-tumor agents

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ABSTRACT

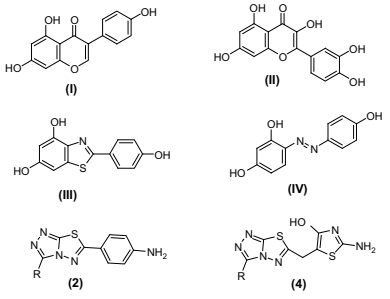
A new series of 3,6-disubstituted triazolo[3,4-b]thiadiazole derivatives have been synthesized by simple, high yielding routes. The key step in the construction of the triazolo[3,4-d]thiadiazole nucleus involves the reaction of 4-amino-5-substituted [1,2,4]triazole-3-thiol with carbon disulphide, 4-amino benzoic acid, (2-amino[1,3]thiazole-4-one-5-yl) acetic acid, and (1H-pyrazolo[3,4-d]pyrimidine-2,4-dithione-5-yl) acetonitrile. The newly synthesized compounds were evaluated for their cytotoxic activity against a panel of 60 human cancer cell lines by the National Cancer Institute (NCI) and some of them demonstrated inhibitory effects on the growth of a wide range of cancer cell lines generally at 10^{-5} M level and in some cases at 10^{-7} M concentrations. In this assay, the anti-tumor activity of the newly synthesized compounds could not be interpreted in terms of tyrosine kinase inactivation but more likely as a relatively broad specificity for the ATP-binding domain of other kinases. The pharmacological mechanism of action for these intriguing compounds has not, as yet, been successful.

1. Introduction

The isoflavone genistein (**I**) [1] and the flavone quercetin (**II**) [2] are competitive inhibitors at the ATP-binding site of kinases, and synthetic flavones modeled on quercetin have been investigated recently in an effort to identify compounds with greater inhibitory selectivity towards tyrosine kinases over serine–threonine kinases [3,4].

Protein tyrosine kinases occupy a central position in the control of cellular proliferation. Several transforming oncogenes (e.g. Src, abl) are known to possess tyrosine kinase activity, and it is well recognized that the response of many cells to growth factors is initiated by the activation of receptor tyrosine kinases (RTKs). Overexpression of certain RTKs shows association with promotion and maintenance of malignant disease [5]. Thus, inactivation of the specific tyrosine kinases that are responsible for the malignant phenotype of certain cancers represents a potential approach for the design of antiproliferative drugs [6]. Some benzothiazoles were designed as potential tyrosine kinase inhibitors modeled on structural comparison with the natural products which compete at the ATP-binding sites of tyrosine kinases [7]. The most interesting bioactive compounds were 4,6-dihydroxy-2-(4-hydroxyphenyl)benzothiazole (**III**) and the related acyclic structure 2,4,4′-trihydroxyazobenzene (**IV**) which have the same overall substitution pattern as genistein.

During the course of this work, we prepared 4-(3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl) aniline derivatives (**2**), 2-amino-5-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl methyl-1, 3-thiazol-4-ol derivatives (**4**) and triazolo[3,4-b]thiadiazole derivatives as analogues to anti-tumor benzothiazoles to compare between benzothiazole nucleus and triazolothiadiazole nucleus and optimize the biological response of the new lead triazolothiadiazole derivatives. The newly synthesized compounds were evaluated for their cytotoxic activity against a panel of 60 human cancer cell lines by the National Cancer Institute.



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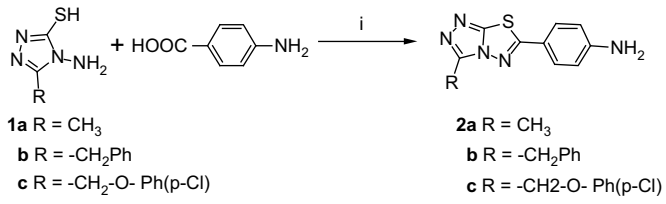


Scheme 1. Reagents and conditions (i) PPA, heat at 200 °C.

2. Results and discussion

A one pot synthesis of 4-(3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl) aniline derivatives (**2**) was achieved when 4-amino-5-substituted [1,2,4]triazole-3-thiol (**1**) [8,9] and 4-amino benzoic acid were heated at 180–200 °C in PPA. The reaction temperature appeared to be crucial for this one-step process. When the reaction was carried out below 180 °C, no intramolecular cyclization was observed, and the starting materials were obtained as the major product (Scheme 1).

We also discovered that 2-amino(5-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)-1,3-thiazol-4-ol derivatives (**4**) could be prepared in one-step synthesis by the reaction of compound (**1**) and (2-amino[1,3]thiazol-4-one-5-yl)acetic acid (**3**) [10] in PPA at 100 °C. When the reaction temperature was raised above 100 °C (130–180 °C) the yield was reduced dramatically (Scheme 2).

To examine further the effect of alteration at the C-6 position of triazolothiadiazoles, we synthesized 3-(3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)methyl-1H-pyrazolo[3,4-d]pyrimidine-4,6-dithione derivatives (**6**) by the reaction of 4-amino-5-substituted [1,2,4]triazole-3-thiol (**1**) with (1H-pyrazolo[3,4-d]pyrimidine-4,6-dithione-3-yl) acetonitrile (**5**) in PPA at 100 °C for 2 h. When the reaction was carried out by fusion of the starting materials, no product was isolated (Scheme 3).

To complete the synthetic work, we prepared the sulfur and nitrogen derivatives at C-6 to examine their effects in biological profile. 3-Substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole-6-thiol (**7**) was prepared by the reaction of 4-amino-5-substituted [1,2,4]triazole-3-thiol (**1**) with carbon disulphide in refluxing pyridine. The disulfide derivatives (**8**) were prepared from the reaction of compound (**7**) with iodine in the presence of sodium carbonate. Also, alkylation of compound (**7**) with alkyl halides takes place readily in the presence of alkaline to give S-alkyl derivatives (**9**), which reacted with hydrazine hydrate to give 6-hydrazino-3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**10**) (Scheme 4).

3. Pharmacology

3.1. Biochemical assay

The biochemical assay of the newly synthesized compounds as CDK inhibitors was determined and expressed as IC_{50} (μM) (Table 1).

The data in Table 1 show that compounds **2a**, **2c**, **4a**, **4c**, **7c**, **8c** showed modestly potent ($IC_{50} < 10$ μM) and the other compounds did not show any activity against CDKs.

3.2. In vitro

Evaluation of anticancer activity on [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole derivatives was performed at the National Cancer Institute (NCI). First the newly synthesized compounds have been evaluated in primary anticancer assay at 10^{-5} M concentration against three cell lines MCF7 (Breast), NCI-H460 (Lung) and SF-268 (CNS) cell lines Table 2.

For NCI criteria compounds, which reduced the growth of any one of the cell lines to ca. 32% or less, are passed on for evaluation in the full panel of 60 human tumor cell lines. The anticancer activity of each compound is deduced from dose–response curves and is presented in Table 2 according to the data provided by NCI. The response parameters GI_{50}, TGI refer to the drug concentration that produced 50% inhibition and total growth inhibition respectively, and are expressed in micromolar concentrations (μM).

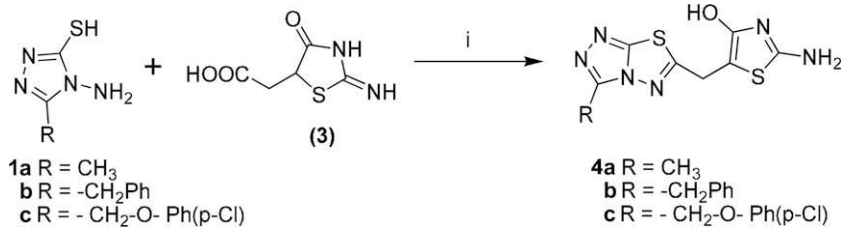
From the analysis of data reported in Table 3, where the activity of compounds **2a**, **2c**, **4a**, **4c**, **7c**, and **8c** is stated, we can evince that compounds **2c** and **4c** maintained the highest growth inhibition activity at micromolar concentrations in different human tumor cell lines. Compound **8c** maintained the moderate cytotoxic activity and finally, compounds **2a**, **4a**, and **7c** maintained the lowest growth inhibition activity against different human tumor cell lines. Compound **4c** exhibited the highest sensitivity against Renal, Colon and Melanoma Cancer cell lines, the best results being against Renal Cancer A498 cell line with log GI_{50} −7.27. The compound **2c** displayed high activity against NCI-H226 (log GI_{50} −5.14) cell line of Non-small cell lung cancer sub-panel and against CCRF-CEM (log GI_{50} −5.0) cell line of Leukemia sub-panel. Compound **8c** exhibited moderate sensitivity against Leukemia SR cell line with log GI_{50} −4.85. Finally, compounds **2a**, **4a** and **7c** displayed the lowest sensitivity against CNS, Non-small cell lung and Colon cancer with log GI_{50} −4.67, −4.11 and −4.09 respectively. Compounds **4c**, **2c** and **8c** were selected for in vivo screening test by the biological committee of NCI.

3.3. In vivo

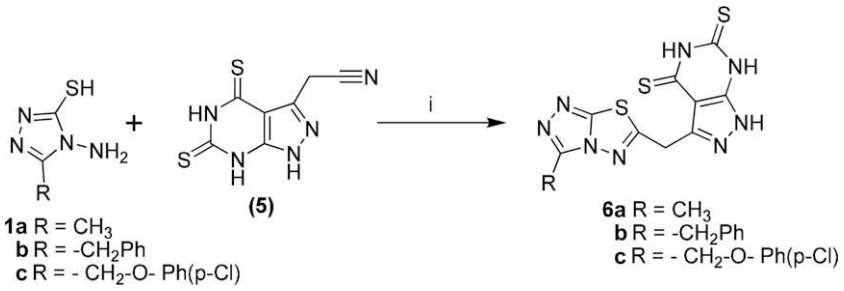
On the basis of the in vitro results three compounds (**2c**, **4c** and **8c**) were selected for *in vivo* anti-tumor activity assays in nude mice xenografted with HCT-116 tumors. Initially acute toxicity in mice was performed for each individual compound, despite their relative high cytotoxic activity in vitro. Compounds (**2c** and **8c**) had no significant cytotoxic activity in mice, but compound (**4c**) showed acute cytotoxicity. Therefore, National Cancer Institute (NCI) selected it for testing *in vivo* anti-tumor Hollow Fiber Assays.

3.3.1. Hollow fiber assay for preliminary in vivo testing

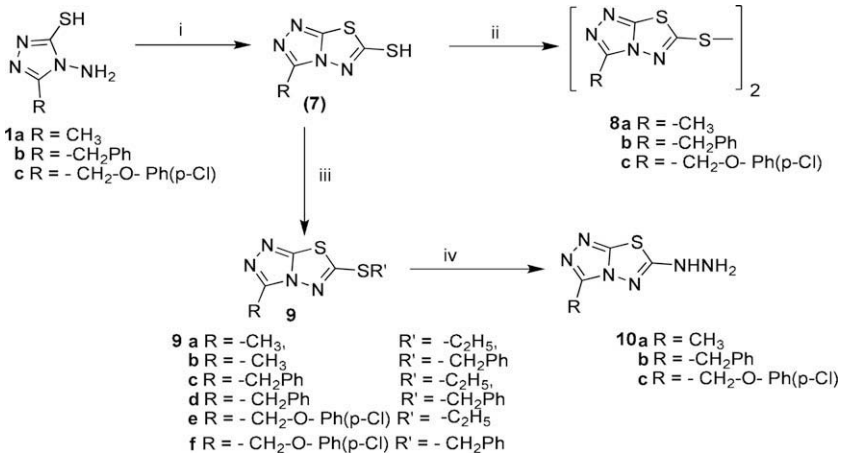
The three compounds (**2c**, **4c** and **8c**) were tested against a minimum of 12 human cancer cell lines. This represents four



Scheme 2. Reagents and conditions (i) PPA, heat at 100 °C.



Scheme 3. Reagents and conditions (i) PPA, heat at 100 °C.



Scheme 4. Reagents and conditions (i) CS_2, pyridine, reflux; (ii) I_2, Na_2CO_3, stirring; (iii) R′X, alcohol, Na_2CO_3, stirring; (iv) $H_2NNH_2 \cdot H_2O$, ethyl alcohol, reflux.

experiments since each experiment contains 3-cell lines. The data are reported as percentage T/C for each of the 2 compound doses against each of the cell lines with separate values calculated for the intraperitoneal and subcutaneous samples. Compounds were found to be active if their combined IP + SC score > 20, and SC score > 8 or a net cell kill of one or more cell lines is referred for xenograft testing. The data of Hollow Fiber Assay for the selected compound (**4c**, **NSC** 723448) are shown in Table 4.

Table 1
IC_{50} Values for the newly synthesized compounds as CDK inhibitors.

Compound	IC_{50} (μM)		
	CDK1/B	CDK2/A	CDK4/D
2a	>120	0.57	>120
2b	>120	>28	14
2c	6.5	0.33	2.1
4a	12	0.25	12
4b	>120	14	>48
4c	0.91	0.18	3
6a	>48	24	10
6b	14	24	>48
6c	29	37	>48
7a	19	6.8	23
7b	25	23	37
7c	0.9	0.40	0.4
8a	6.8	23	11
8b	5.3	15	25
8c	11	0.82	8.0
9a	>120	11	14
9b	>48	16	>120
9c	>120	24	>48
9d	25	>28	18
9e	>48	24	>120
9f	>120	>28	37
10a	22	11	>120
10b	>120	>28	>48
10c	28	15	>120

4. Conclusion

From the present study, it can be concluded that the newly synthesized compounds were designed to act as tyrosine kinases but they were found to be inactive in terms of tyrosine kinase inactivation. Their mode of action could be interpreted more likely as a relatively broad specificity for the ATP-binding domain of other kinases. Also, the anti-tumor activity of [1,2,4]triazolo[3,4-b][1,3,4]thiadiazoles appears to be related to some structural requirements and to the presence of particular substituents, as matter of fact 4-chlorophenoxymethylene moiety plays an important role for anti-tumor activity. These compounds were not active as PTKs so we tested them as CDKs

Table 2
Results of primary assay of anti-tumor activity of the newly synthesized compounds on 3-cell lines.[a]

Compound	Growth percentage at 10^{-5} M concentration		
	Cell line (cancer)		
	MCF7 (Breast)	NCI-H460 (Lung)	SF-268 (CNS)
2a	119	null	null
2c	128	null	null
4a	130	null	null
4c	null	null	null
7c	null	null	null
8c	147	null	null

[a] We reported the active compounds only.

Table 3
Inhibition of cancer cell lines by compounds **2a**, **2c**, **4a**, **4c**, **7c** and **8c**.

Cell line	Cytotoxicity log GI_{50}[a] (M)					
	2a	**2c**	**4a**	**4c**	**7c**	**8c**
Leukemia						
CCRF-CEM	N[b]	−5.0	N	NT[c]	−4.01	−4.12
RPMI-8226	N	−4.11	NT	N	N	−4.35
SR	N	−4.04	N	−4.31	−4.02	−4.85
Non-small cell lung						
HOP-62	−4.06	−4.69	N	N	N	−4.18
HOP-92	NT	−4.01	N	−4.01	−4.1	−4.74
NCI-H226	N	−5.14	−4.11	NT	−4.05	−4.06
NCI-H322M	N	N	−4.06	−4.95	N	NT
Colon						
HCC-2998	N	N	N	N	N	−4.79
HCT-116	NT	−4.46	−4.08	NT	−4.0	N
HCT-15	N	NT	N	−6.13	NT	−4.06
KM12	−4.0	−4.0	−4.01	−5.13	N	N
CNS						
SF-268	N	−4.33	N	N	N	N
SF-539	N	−4.46	N	−4.4	N	N
SNB-75	−4.67	NT	N	N	N	N
Melanoma						
LOXIMVI	N	N	N	−5.44	N	NT
UACC-62	N	N	N	−4.3	N	−4.35
Ovarian						
OVCAR-3	N	N	N	N	−4.09	−4.45
OVCAR-4	N	N	N	NT	N	−4.33
Renal						
A498	N	N	N	−7.27	N	−4.54
ACHN	N	−4.27	N	−4.45	N	N
CAKI-1	N	−4.31	−4.2	N	N	−4.11
TK-10	NT	−4.07	N	−4.34	N	−4.14
Breast						
MCF7	N	−4.39	N	−4.0	N	−4.35
HS578T	−4.05	−4.06	N	−4.5	−4.06	N
BT-549	−4.0	NT	N	N	−4.1	−4.32
Mean value[d]	−4.16	−4.36	−4.09	−5.75	−4.05	−4.36

[a] Data obtained from NCI's in vitro disease-oriented tumor cells screen.
[b] In active.
[c] Not tested.
[d] Mean value over all active cell lines tested.

(ATP competitive inhibitors). We found that a large hydrophobic group should be found at position 4 to give a good biological activity. Compounds with thiazole-ring were found to be active because we think that the thiazole-ring came at the sugar pocket of CDKs protein. The pharmacological mechanism of action for these intriguing compounds is under investigation.

5. Experimental

5.1. Pharmacology

5.1.1. Anticancer assay for preliminary in vitro testing

The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cell suspensions that were diluted according to the particular cell type and the expected target cell density (5000–40,000 cells per well based on cell growth characteristics) were added by pipet (100 μl) into 96-well microtiter plates. Inoculates were allowed a pre-incubation period of 24 h at 37 °C for stabilization. Dilution at twice the intended test concentration was added at time zero in 100-μl aliquots to the microtiter plate wells. Usually, test compounds were evaluated at five 10-fold dilutions. In a routine testing, the highest well concentration is 10E-4 M, but for the standard agents the highest well concentration used depended on the agent. Incubation lasted for 48 h in 5% CO_2 atmosphere and 100% humidity. The cells were assayed by using the sulforhodamine B assay [11]. A plate reader was used to read the optical densities [12,13].

Table 4
The IP, SC and cell kill data for compound 4c.

Exp id			
	HF: 876	IP score	11
	HF: 877	SC score	15
	HF: 878	Total score	26
	HF: 879	Cell kill	N

IP: intraperitoneal; SC: subcutaneous.

5.1.2. Hollow fiber assay for preliminary in vivo testing

In this assay the human tumor cells are cultivated in polyvinylidene fluoride (PVDF) hollow fibers, and a sample of each cell line is implanted into each of two physiologic compartments (intraperitoneal and subcutaneous) in mice. Each test mouse receives six fibers (3 intraperitoneally and 3 subcutaneously) representing 3 distinct cancer cell lines. Three mice are treated with potential anti-tumor compounds at each of two test doses by the intraperitoneal route using a QD × 4 treatment schedule. Vehicle controls consist of six mice receiving the compound diluents only. The fiber cultures are collected on the day following the last day of treatment. To assess anticancer effects, viable cell mass is determined for each of the cell lines using a formazan dye (MTT) conversion assay. From this, the percentage T/C can be calculated using the average optical density of the compound treated samples divided by the average optical density of the vehicle controls. In addition, the net increase in cell mass can be determined for each sample as a sample of fiber cultures is assessed for viable cell mass on the day of implantation into mice. Thus, the cytostatic and cytocidal capacities of the test compound can be assessed [13].

5.2. Chemistry

All melting points are uncorrected and determined by the open capillary method. IR spectra were recorded using (KBr) disc. ^{1}H NMR spectra were recorded using DMSO-d_6 as a solvent and TMS as an internal reference. Chemical shifts are expressed in δ units (ppm). Mass spectra were recorded with a mass spectrometer MS9 (AEI) 70 eV. Elemental combustion analyses were within ±0.4% of the theoretical values. All the results were in an acceptable range.

5.2.1. General procedure for the synthesis of 4-(3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl) anilines **2**

A mixture of 4-amino-5-substituted [1,2,4]triazole-3-thiol (**1**) (6.81 mmol) and polyphosphoric acid (20 ml) was heated to 50–60 °C with stirring, then 4-aminobenzoic acid (6.9 mmol) added portionwise. The mixture was heated at 180–200 °C for 3 h with stirring then poured onto ice. After neutralizing with concentrated aqueous ammonia solution, the crude product was collected by filtration, and recrystallized from appropriate solvent to give 4-(3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl) anilines derivatives (**2**).

*5.2.1.1. 4-(3-Methyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl) aniline **2a**.* Compound **2a** was obtained as green crystals (80% yield); recrystallized from ethyl alcohol; M.P. 204–206 °C; IR, 3320–3400, 2995, 1600 cm^{-1}; ^{13}C NMR (DMSO-d_6) δ = 15, 120, 130, 140, 146, 150, 153, 159; ^{15}NNMR (DMSO-d_6) δ = −60.2, −68.1, −80.4, −120.5, −311.6; M.S, m/z 232 (M^+, 10%), 139 (21.9%), 113 (100%), 81 (40%), and 66 (35.2%). Anal. ($C_{10}H_9N_5S\cdot 0.25H_2O$) C, H, N.

*5.2.1.2. 4-(3-Benzyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl) aniline **2b**.* Compound (**2b**) was obtained as yellowish white crystals (85% yield); recrystallized from ethyl alcohol; M.P. 180–183 °C;

^{1}H NMR (DMSO-d_6), $\delta = 7.9–7$ (m, 9H), 6.3 (s, 2H), 4 (s, 2H); M. S, m/z 307 (M^+, 100%), 215 (14%), 189 (35%), 157 (20%), and 66 (15.11%). Anal. ($C_{16}H_{13}N_5S$) C, H, N.

5.2.1.3. 4-{3-[(4-Chlorophenoxy)methyl][1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl} aniline ***2c***. Compound (**2c**) was obtained as buff crystals (76% yield); recrystallized from ethyl alcohol; M.P. 166–168 °C; ^{1}H NMR (DMSO-d_6), $\delta = 7.5–6.9$ (m, 8H), 5.3 (s, 2H), and 4.8 (s, 2H). Anal. ($C_{16}H_{12}ClN_5OS \cdot 0.5H_2O$) C, H, N.

5.2.2. General procedure for the synthesis of 2-amino-5-[(3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)methyl]-1,3-thiazol-4-ol ***4***

A mixture of (2-amino[1,3]thiazole-4-one-5-yl)acetic acid (**3**) (6.81 mmol) and polyphosphoric acid (20 ml) was heated to 50 °C with stirring, then 4-amino-5-substituted [1,2,4]triazole-3-thiol (**1**) (6.81 mmol) was added portionwise. The reaction mixture was heated at 100 °C for 4 h with stirring, then cooled, and poured into ice-cold 10% aqueous sodium carbonate. The solid product was collected, washed with water, and dried to give compound (**4**) that was purified by recrystallization from the appropriate solvent.

5.2.2.1. 2-Amino-5-[(3-methyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)methyl]-1,3-thiazol-4-ol ***4a***. Compound **4a** was obtained as brown crystals (65% yield); recrystallized from DMF/ethyl alcohol; M.P. 260–262 °C; IR, 3390–3350, 2980, 1680, 1620 cm^{-1}, ^{1}H NMR (DMSO-d_6), $\delta = 6.1$ (s, 1H), 4.5 (s, 2H), 3.4 (d, 2H), 1.3 (s, 3H). Anal. ($C_8H_8N_6OS_2 \cdot 0.25H_2O$) C, H, N.

5.2.2.2. 2-Amino-5-[(3-benzyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)methyl]-1,3-thiazol-4-ol ***4b***. Compound **4b** was obtained as dark brown crystals (71% yield); recrystallized from ethyl alcohol; M.P. 210–212 °C; ^{13}C NMR (DMSO-d_6) $\delta = 30$, 33, 100, 126, 128, 130, 134, 143, 156, 165, 168, 180; ^{15}N NMR (DMSO-d_6) $\delta = -55.2$, −60.1, −78.4, −118.5, −180.1, −299.6; M.S, m/z 344 (11%), 253 (41%), 237 (3.15%), 138 (15.5%), 124 (20.5%), and 98 (35.71%). Anal. ($C_{14}H_{12}N_6OS_2$) C, H, N.

5.2.2.3. 2-Amino-5-({3-[(4-chlorophenoxy)methyl][1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl}-methyl)-1,3-thiazol-4-ol ***4c***. Compound **4c** was obtained as buff powders (59% yield); recrystallized from ethyl alcohol; M.P. 200–202 °C; ^{1}H NMR (DMSO-d_6), $\delta = 5.9$ (s, 1H), 7.3–6.9 (m, 4H), 5.4 (s, 2H), 4.8 (s, 2H) and 3.4 (s, 2H); M.S, m/z 394.5 (5.3%), 253 (44.5%), 237 (2.5%), 138 (20.1%), and 98 (100%). Anal. ($C_{14}H_{11}ClN_6O_2S_2 \cdot 0.5H_2O$) C, H, N.

5.2.3. General procedure for the synthesis of 3-[(3-substituted [1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazol-6-yl)methyl]-1H-pyrazolo[3,4-d]pyrimidine-4,6-dithione ***6***

A mixture of (1H-pyrazolo[3,4-d]pyrimidine-2,4-dithione-5-yl)acetonitrile (**5**) (6.81 mmol) and polyphosphoric acid (20 ml) was heated at 100 °C with stirring, then 4-amino-5-substituted [1,2,4]triazole-3-thiol (**1**) (6.81 mmol)was added. The reaction mixture was maintained at this temperature for 2 h with stirring, then cooled and poured into ice-cold 10% aqueous sodium bicarbonate. The solid product was collected by vacuum filtration, washed with water and recrystallized from appropriate solvent to give compound (**6**).

5.2.3.1. 3-[(3-Methyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)methyl]-1H-pyrazolo[3,4-d]-pyramidine-4,6-dithione ***6a***. Compound **6a** was obtained as yellow crystals (89% yield); recrystallized from DMF; M.P. 240–242 °C; ^{1}H NMR (DMSO-d_6), $\delta = 13.4$ (s, 1H), 11.4 (s, 1H), 10.9 (s, 1H), 3.5 (s, 2H), and 1.4 (s, 3H); ^{13}C NMR (DMSO-d_6) $\delta = 15$, 25, 110, 143, 148, 152, 155, 158, 163, 182; ^{15}N NMR (DMSO-d_6) $\delta = -58.2$, −65.1, −80.4, −120.5, −130.2, −180.1, −260.6, −320.6. Anal. ($C_{10}H_8N_8S_3 \cdot 1H_2O$) C, H, N.

5.2.3.2. 3-[(3-Benzyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)methyl]-1H-pyrazolo[3,4-d]-pyramidine-4,6-dithione ***6b***. Compound **6b** was obtained as yellow crystals (77% yield); recrystallized from DMF; M.P. 230–232 °C; IR, 3400, 3120, 2995, 1600, 1200 cm^{-1}; M.S, m/z 413 (M^+, 2.77%), 321 (17.7%), 262 (20.05%), 203 (40.61%), 138 (10.74%), 124 (32.0%), 98 (100%), and 66 (5.45%). Anal. ($C_{16}H_{12}N_8S_3 \cdot 0.25H_2O$) C, H, N.

5.2.3.3. 3-({3-[(4-chlorophenoxy)methyl][1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)methyl]-1H-pyrazolo[3,4-d] pyramidine-4,6-dithione ***6c***. Compound **6b** was obtained as yellow crystals (75% yield); recrystallized from DMF; M.P. 238–240 °C; ^{1}H NMR (DMSO-d_6), $\delta = 13.4$ (s, 1H), 11.9 (s, 1H), 10.5 (s, 1H), 7.5–7.0 (m, 4H), 5.4 (s, 2H), and 3.8 (s, 2H). Anal. ($C_{16}H_{11}ClN_8OS_3 \cdot 0.5H_2O$) C, H, N.

5.2.4. General procedure for the synthesis of 3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole-6-thioles ***7***

A solution of 4-amino-5-substituted [1,2,4]triazole-3-thiol (**1**) (0.01 mol) in pyridine (20 ml) was treated with carbon disulfide (2 ml) and few drops of triethylamine. The reaction mixture was refluxed at 100 °C with stirring for 6 h, then cooled, poured into ice-cold water and makes the reaction mixture slightly acidic by adding HCl. The solid product was collected by vacuum filtration, washed well with water and recrystallized from the appropriate solvent to give the desired compound (**7**).

5.2.4.1. 3-Methyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole-6-thiol ***7a***. Compound **7a** was obtained as shiny white crystals (82% yield), recrystallized from ethyl alcohol; M.P. 180–182 °C; M.S, m/z 172 (100%), 139 (20%), 113 (44.55%), 81 (14.82%), and 66 (30.04%). Anal. ($C_4H_4N_4S_2$) C, H, N.

5.2.4.2. 3-Benzyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole-6-thiol ***7b***. Compound **7b** was obtained as shiny buff (88% yield); recrystallized from ethyl alcohol; M.P. 192–194 °C; ^{1}H NMR (DMSO-d_6), $\delta = 7.3–7.1$ (m, 5H), 4.9 (s, 1H), and 4.3 (s, 2H). Anal. ($C_{10}H_8N_4S_2$) C, H, N.

5.2.4.3. 3-[(4-Chlorophenoxy)methyl][1,2,4]triazolo[3,4-b][1,3,4]thiadiazole-6-thiol ***7c***. Compound **7c** was obtained as white crystals (97% yield); recrystallized from ethyl alcohol; M.P. 166–168 °C; IR, 3190, 2990, 1610, 1200 and 1100 cm^{-1}; ^{1}H NMR (DMSO-d_6), $\delta = 7.4–6.9$ (m, 4H), 5.4 (s, 2H), and 3.5 (s, 1H). Anal. ($C_{10}H_7ClN_4OS_2 \cdot 0.25H_2O$) C, H, N.

5.2.5. General procedure for the synthesis of 3-substituted-6-[(3-substituted [1,2,4]triazolo-[3,4-b][1,3,4]thiadiazol-6-yl) dithio][1,2,4]triazolo[3,4-b][1,3,4]thiadiazole derivatives ***8***

A suspension of compound (**7**) (0.01 mol) in 10% Na_2CO_3 solution (20 ml) was treated with iodine (0.01 mol) portionwise. The reaction mixture was stirred at room temperature for 5 h, and then the solid so formed was collected by vacuum filtration, washed well with water and dried at 50 °C for 6 h. The desired compound (**8**) was obtained after recrystallization from ethyl alcohol.

5.2.5.1. 3-Methyl-6-[(3-methyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)dithio][1,2,4] triazolo[3,4-b][1,3,4]thiadiazole ***8a***. Compound **8a** was obtained as shiny white crystals (65% yield); M.P. 160–162 °C; ^{13}C NMR ($CDCl_3$) $\delta = 15$, 143, 146, 153; m/z 342 (2.09%), 171 (100%), 113 (36.16%), 81 (57.15%), and 66 (10.70%); ^{15}N NMR ($CDCl_3$) $\delta = -55.2$, −62.1, −70.4, −118.5. Anal. ($C_8H_6N_8S_4 \cdot 0.5H_2O$) C, H, N.

5.2.5.2. 3-Benzyl-6-[(3-benzyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)dithio][1,2,4] triazolo[3,4-b][1,3,4]thiadiazole ***8b***. Compound **8b** was obtained as shiny pale brown crystals (60% yield); M.P. 170–172 °C; M.S, m/z 494 (1.09%), 274 (100%), 215 (10.20%), 189 (33.30%), 98 (15.08%), and 66 (41.15%). Anal. ($C_{20}H_{14}N_8S_4 \cdot 0.25H_2O$) C, H, N.

*5.2.5.3. 3-[(4-Chlorophenoxy)methyl]-6-({3-[(4-chlorophenoxy)methyl][1,2,4]triazolo-[3,4-b][1,3,4]thiadiazol-6-yl}dithio)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **8c**.* Compound **8c** was obtained as metallic purple crystals (58% yield); M.P. 155–157 °C; ^{1}H NMR (DMSO-d_6), $\delta = 7.7–7.1$ (m, 8H), and 5.2 (s, 4H). Anal. ($C_{20}H_{12}Cl_2N_8O_2S_4 \cdot 0.5H_2O$) C, H, N.

*5.2.6. General procedure for the synthesis of 6-alkylthio-3-substituted [1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole derivatives **9***

To a stirred solution of 3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole-6-thiole (**7**) (0.01 mol) in ethyl alcohol (10 ml) at room temperature was added 1 N NaOH (15 ml), then the reaction mixture was treated with appropriate alkyl halide (0.01 mol) drop-wise with stirring. The reaction mixture was stirred for 4 h, and then the solid product was collected by filtration, washed with water and diethyl ether to give the titled compound (**9**).

*5.2.6.1. 6-(Ethylthio)-3-methyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **9a**.* Compound **9a** was obtained as yellow crystals (90% yield); recrystallized from ethyl alcohol; M.P. 110–112 °C; M.S, m/z 200 (10%), 139 (7.77%), 113 (30.60%), 81 (100%), and 66 (59.78%). Anal. ($C_6H_8N_4S_2$) C, H, N.

*5.2.6.2. 6-(Benzylthio)-3-methyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **9b**.* Compound **9b** was obtained as yellow crystals (92% yield); recrystallized from ethyl alcohol; M.P. 98–100 °C; ^{13}C NMR (DMSO-d_6) $\delta = 14$, 35, 125, 127, 129, 135, 145, 149, 156; M.S, m/z 262 (5%), 171(20.1%), 139 (42.2%), 113 (30%), 81 (100%) and 55 (66.1%); ^{15}N NMR (DMSO-d_6) $\delta = -58.2$, −60.1, −74.4, −120.5. Anal. ($C_{11}H_{10}N_4S_2$) C, H, N.

*5.2.6.3. 3-Benzyl-6-(ethylthio)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **9c**.* Compound **9c** was obtained as yellow crystals (85% yield); recrystallized from ethyl alcohol; M.P. 115–118 °C; ^{1}H NMR (DMSO-d_6), $\delta = 7.3–6.9$ (m, 5H), 4.2 (s, 2H), 2.1–2 (q, 2H) and 1–1.3 (t, 3H); Anal. ($C_{12}H_{12}N_4S_2$) C, H, N.

*5.2.6.4. 3-Benzyl-6-(benzylthio)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **9d**.* Compound **9d** was obtained as yellow crystals (88% yield); recrystallized from ethyl alcohol; M.P. 108–110 °C;, ^{1}H NMR (DMSO-d_6), $\delta = 7.6–6.9$ (m, 10H), 4.6 (s, 2H) and 4.1 (s, 2H); Anal. ($C_{17}H_{14}N_4S_2 \cdot 0.25H_2O$) C, H, N.

*5.2.6.5. 3-[(4-Chlorophenoxy)methyl]-6-(ethylthio)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **9e**.* Compound **9e** was obtained as yellow crystals (80% yield); recrystallized from ethyl alcohol; M.P. 120–122 °C; ^{1}H NMR (DMSO-d_6), $\delta = 7.4–7.0$ (m, 4H), 5.3 (s, 2H), 2.1–1.9 (q, 2H), and 1.1–0.9 (t, 3H). Anal. ($C_{12}H_{11}ClN_4OS_2 \cdot 0.75H_2O$) C, H, N.

*5.2.6.6. 6-(Benzylthio)-3-[(4-Chlorophenoxy)methyl][1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **9f**.* Compound **9f** was obtained as yellow crystals (75% yield), recrystallized from ethyl alcohol; M.P. 105–107 °C; ^{1}H NMR (DMSO-d_6), $\delta = 7.6–7.2$ (m, 9H), 5.1 (s, 2H) and 4.4 (s, 2H); Anal. ($C_{17}H_{13}ClN_4OS_2$) C, H, N.

*5.2.7. General procedure for the synthesis of 6-hydrazino-3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazoles **10***

To a solution of 6-alkylthio-3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**9**) (0.01 mol) in ethyl alcohol (25 ml) was added hydrazine hydrate (0.015 mol). The reaction mixture was refluxed on water-bath for 3 h, and then the excess of alcohol was removed under reduced pressure. The solid residue was triturated with water and 2 drops of acetic acid, the solid precipitate was collected by filtration, washed well with water and recrystallized from alcohol to give compound (**10**).

*5.2.7.1. 6-Hydrazino-3-methyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **10a**.* Compound **10a** was obtained as white crystals (50% yield); M.P.125–127 °C; IR, 3400, 3300–3340, 2995 cm^{-1}; ^{1}H NMR (DMSO-d_6), $\delta = 4.7–4.6$ (t, 1H), 4.6–4.5 (d, 2H) and 1.7 (s, 3H); M.S, m/z 170 (100%), 139 (30.05%), 113 (20.70%), 81(10.05%), and 66 (14.40%). Anal. ($C_4H_6N_6S \cdot 0.25H_2O$) C, H, N.

*5.2.7.2. 3-Benzyl-6-hydrazino[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **10b**.* Compound **10b** was obtained as white crystals (65% yield); M.P. 130–132 °C; ^{1}H NMR (DMSO-d_6), $\delta = 7.2–6.9$ (m, 5H), 4.8–4.7 (t, 1H), 4.5–4.4 (d, 2H), and 4.2 (s, 2H); ^{13}C NMR (DMSO-d_6) $\delta = 30$, 126, 128, 130, 136, 146, 151, 158; Anal. ($C_{10}H_{10}N_6S \cdot 1H_2O$) C, H, N.

*5.2.7.3. 3-[(4-Chlorophenoxy)methyl]-6-hydrazino[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **10c**.* Compound **10c** was obtained as white crystals (45% yield); M.P. 118–120 °C; ^{1}H NMR (DMSO-d_6), $\delta = 7.5–7$ (m, 4H), 5.4 (s, 2H), 4.9–4.7 (t, 1H), and 4.7 (d, 2H); ^{15}N NMR (DMSO-d_6) $\delta = -54.2$, −62.1, −72.4, −122.5, −220.4, −303.2; M.S, m/z 296.5 (20%), 169 (3.04%), 155 (100%), 124 (19.91%), 98 (40.41), and (22.11%). Anal. ($C_{10}H_9ClN_6OS \cdot 0.75H_2O$) C, H, N.

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