Evaluation of Antiproliferative Effect *in Vitro* of Some 2-Amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole Derivatives

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New compounds of *N*-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole set were synthesized and tested for their antiproliferative activity as part of our research in the antitumour field. Title compounds were obtained by reaction of sulfinylbis(2,4-dihydroxythiobenzoyl) (STB) with 4-substituted 3-thiosemicarbazides. The structures of compounds were identified from elemental, IR, ^{1}H-, ^{13}C-NMR and MS spectra analyses. The cytotoxicity *in vitro* against human bladder cancer HCV29T cells was determined. The most active compounds were also tested against human cancer cell lines: SW707 (rectal), A549 (lung) and T47D (breast). The antiproliferative effect of some compounds was higher than cisplatin studied comparatively.

Key words 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole derivative; synthesis; antiproliferative activity; *in vitro* study

A great number of 1,3,4-thiadiazole derivatives display interesting antitumour activities *iv vitro* and *in vivo* conditions.[1—10] Their mechanism of action is different for various types of substitution of 1,3,4-thiadiazole ring.[11—15] Very promising for anticancer therapy seems to be the action connected with the apoptotic mechanisms and angiogenesis, which is a crucial step in the tumorgenesis.[16—20]

Beside on this consideration we elaborated a new ring-forming method for the synthesis of *N*-substituted 2-amino 5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles as a part of our antitumour and antifungal search program of compounds with resorcinol moiety.[21] The presence of 2,4-dihydroxyphenyl substituent in the molecule is responsible first of all for its amphiphilic character. However, intensification of aminothiadiazole pharmacophore function and possibility of additional interactions *via* hydrogens bonds or van der Waal's forces with the potential molecular target can not be excluded. Moreover, relatively low level of toxicity of derivatives with 2,4-dihydroxyphenyl moiety is registered.[22,23]

Recently, we have reported the synthesis and antiproliferative activity *in vitro* of some *N*-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole analogues.[24,25] It was found that *N*-alkyl and *N*-morpholinoalkyl derivatives exhibit significantly lower effect than phenyl ones. Some compounds showed stronger cytotoxic effect than cisplatin studied comparatively. The highest antiproliferative activity was found for the compounds with hydrophobic substituents ($\pi>0$) of electronwithdrawing character ($\sigma>0$).[26]

Extending the research in this area, we decided to obtain new derivatives, mainly differently substituted in *N*-aryl ring 2-phenylamino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles. Antiproliferative effect *in vitro* of compounds against panel of human cancer cell lines was established. Influence of *N*-substitution type on antitumour activity is discussed.

Chemistry

The thiadiazole derivatives exemplified in this paper were prepared according to the route described in Fig. 1.[25] Treatment of sulfinylbis(2,4-dihydroxythiobenzoyl) (STB) with appropriate 4-substituted-3-thiosemicarbazides in methanol afforded *N*-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles (II—XIII). 2-Amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (I) was prepared from STB and semicarbazide hydrochloride. STB was obtained from 2,4-dihydroxybenzenecarbodithioic acid and $SOCl_2$ in diethyl ether.[25]

In the ^{1}H-NMR spectrum of compound I the band in the 9.92 ppm corresponding to unsubstituted amine group is registered. The signal of proton of NH group substituted with the alkyl group is shift significantly in the range of higher fields (II). In the range of low fields as a rule there are registered two signals corresponding to protons of OH group in the resorcinol moiety.[25] The resonance signal of C_6–H proton appears in the doublet form in the range about 7.8—7.5 ppm with the conjugation constant $J=8.7$ Hz, and C_3–H in the range 6.5—6.4 with $J=2.3$ Hz. However, C_5–H is registered in the doublet–doublets form at about 6.4—6.3 ppm of conjugation constants $J=8.5$ and 2.3 Hz respectively. In the ^{13}C-NMR spectrum characteristic signals of carbon atoms of 2-amino-1,3,4-thiadiazole ring appear in the range 166—163 ppm and 154—155 ppm.[27]

Mass spectra of compounds gave molecular ion peaks, however, with different intensities. The major fragmentation pathway in the most derivatives involved the cleavage of the S–C_5 and N–N bonds of 1,3,4-thiadiazole ring with formation of $(HO)_2C_6H_3CN^+$ (*m*/*z* 135) and *m*/*z* $[M-135]^+$ ions.[28] The cleavage of C_5–N_4 and S–C_2 bonds directs to $(HO)_2C_6H_3CS^+$ (*m*/*z* 153) fragmentation is also observed.

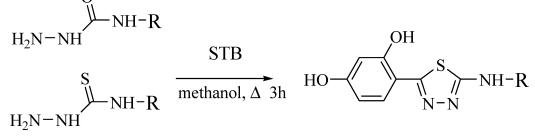

Fig. 1. Synthesis Scheme of *N*-Substituted 2-Amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles

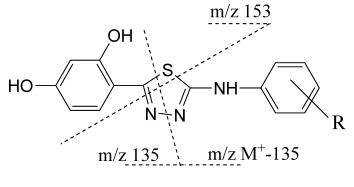

Fig. 2. Fragmentation Pattern of Compounds IV, VI, VII, X, XI, XIII

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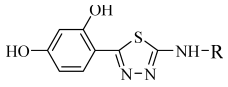
Table 1. Structure and Antiproliferative Activity of *N*-Substituted 2-Amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles against the Cells of Human Cancer Lines HCV29T, SW707, A549 and T47D Expressed as ID_{50} [μM][a)]



No.	Substituent	ID_{50} [μM]			
		HCV29T	SW 707	A 549	T47D
I	H-	205.7±14.3	—[c)]	—[c)]	—[c)]
III	Cyclohexyl-	47.2±2.5	28.9±26.3	48.3±8.5	14.6±4.9
IV	2,4-CH_3-C_6H_3-	81.6±13.4	96.7±6.1	98.5±30.1	19.9±9.5
V	2,5-Cl-C_6H_3-	69.2±2.9	50.6±26.4	27.4±0.7	10.1±0.7
VI	2,6-Cl-C_6H_3-	77.6±2.1	68.1±8.9	54.6±20.1	23.3±2.9
VII	4-Br-C_6H_4-	41.2±12.5	33.7±32.2	24.0±9.4	12.5±1.7
VIII	4-I-C_6H_4-	20.7±2.1	19.5±14.7	17.0±3.0	9.7±0.7
IX	2-CH_3-5-Cl-C_6H_3-	58.9±4.9	34.1±26.8	25.1±7.1	13.4±1.2
X	4-CH_3-3-Cl-C_6H_3-	NEG[b)]	—[c)]	—[c)]	—[c)]
XI	4-CH_3O-C_6H_4-	NEG[b)]	—[c)]	—[c)]	—[c)]
XII	4-$CH_3CH_2OC(=O)$-C_6H_4-	105.3±25.7	—[c)]	—[c)]	—[c)]
XIII	C_6H_5-CH_2-	115.0±22.0	—[c)]	—[c)]	—[c)]
XIV	Cisplatin	2.3±5.0	16.3±5.0	11.0±4.7	20.7±5.0

a) ID_{50} [μM], indicates the compound concentration that inhibits the proliferation rate of tumour cells by 50% as compared to the control untreated cells. The values are the means±S.D. of 9 independent experiments. *b*) NEG: negative in the studied concentrations (up to 100 μg/ml). *c*) — experiment was not performed.

The mass fragmentation pathway of compounds is shown in Fig. 2. Some compounds undergo specific fragmentations. In the case of compound I elimination of isothiocyanic acid, M^+-HNCS is registered, forming *m*/*z* 150 ion. For benzyl derivative (XIII) characteristic, relatively strong signal corresponding to tropylic cation $C_7H_7^+$ (*m*/*z* 91) is observed as the effect of disconnection α and β atoms in relation to the phenyl ring.

Results and Discussion

Compounds of the structure presented in Table 1 have been evaluated for their antiproliferative activity against human bladder cancer HCV29T cells. The cytotoxic activity *in vitro* was expressed as ID_{50} [μM], the concentration of compound that inhibits proliferation rate of the tumour cells by 50% as compared to the control untreated cells. Compounds II were not tested due to of lack of dissolubility in the conditions of biological test. Cisplatin was used as a reference drug. The results of screening are summarized in Table 1.

Parent compound I, with unsubstituted amine group shows weak activity. All *N*-substituted derivatives, except for X and XI, exhibit higher inhibition of the proliferation rate of tumour cells than unsubstituted one (I). Derivatives X and XI did not reveal any cytotoxic activity below concentrations studied. Compound VIII with iodine atom in the *para*-position of the *N*-phenyl ring proves to be the most active against HCV29T cells.

The selected compounds were also tested against A549 cells from human non-small lung carcinoma, against T47D cells from human breast cancer and against SW707 cells from human rectal adenocarcinoma (Table 1). Antiproliferative effect against T47D cells of some tested derivatives is higher than cisplatin. At the same time compounds V and VIII, as the most active derivatives from this set of tested *N*-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles, meet the cytotoxic activity criterion against this line for new antiproliferative drugs ($ID_{50} \leq 4$ μg/ml).[29)] But the activity of compounds against A549 and SW707 cells is lower than cisplatin studied comparatively (Table 1).

Analyzing structure and activity the attention should be paid to a relatively high activity of cyclohexyl derivative (III) (Table 1), whereas acyclic aliphatic derivatives exhibited poor antiproliferative properties.[26)] Activity of isomeric chloromethylphenyl derivatives is differentiated. Compound (IX) is characterized by high antiproliferative effect, but compound X does not exhibit activity on the level of studied concentrations. This indicates an important role of methyl and chlorine substituents position, antagonistic electron effects at retained lipophilicity level. Lack of activity was also found for derivative (XI), with methoxy substituent in the *para*-position of *N*-aryl ring. This is probably caused by a relatively strong electron-donating of $–OCH_3$ substituent effect.

The highest activity for derivatives VII, VIII with bromine or iodine atom in the *para*-position of aryl ring respectively and for 2-methyl-5-chlorophenyl derivative (IX) was found. It confirms our earlier finding about advantageous influence of electronwithdrawing ($\sigma>0$) and hydrophobic ($\pi>0$) substituents of *N*-phenyl ring. In the case of compounds VII and VIII strong polarizability of bromine and iodine atoms should probably be taken into account.

Verification of these hypotheses requires further research of structure–activity type based on various structural parameters and electron ones determined by calculation quantum-chemical methods as well as experimentally estimated lipophilicity parameters and characteristic bands of spectroscopic spectra.

Experimental

Analytical Studies The melting point (mp) was determined on a BUCHI B-540 (Switzerland) melting point apparatus. The elemental analysis was performed in order to determine C, H and N contents (Perkin-Elmer 2400). The analyses (C, H, N) were within ±0.4% of the theoretical values. The vibrational spectra were recorded with a Perkin-Elmer FT-IR 1725X spectrophotometer (in potassium bromide). The spectra were made in the range of 600—4000 cm^{-1}. NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ on a Varian Mercury 400 or Bruker DRX 500 instrument. Chemical shifts (δ, ppm) were given in with tetramethylsilane (TMS). The spectra MS

(EI, 70 eV) were recorded using the apparatus AMD-604.

Bioassays The following established *in vitro* human cell lines were applied: T47D (breast cancer), SW707 (rectal adenocarcinoma), A549 (non-small cell lung carcinoma) from the American Type Culture Collection (Rockville, Maryland, U.S.A.) and HCV29T (bladder cancer) from the Fibiger Institute, Copenhagen, Denmark. Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, U.S.A.) at a density of 10^4 cells/well. All cell lines were maintained in the opti-MEM medium supplement with 2 mM glutamine (Gibco, Warsaw, Poland), streptomycin (50 μg/ml), penicillin (50 U/ml) (Polfa, Tarchomin, Poland) and 5% fetal calf serum (Gibco, Grand Island, U.S.A.). The cells were incubated at 37 °C in the humid atmosphere saturated with 5% CO_2. The solutions of compounds (1 mg/ml) were prepared *ex tempore* by dissolving the substance in 100 μl of DMSO completed with 900 μl of tissue culture medium. Afterwards, the compounds were diluted in the culture medium to reach the final concentrations ranging from 0.1 to 100 μg/ml. The solvent (DMSO) in the highest concentration used in the test did not reveal any cytotoxic activity. Cisplatin was applied as a test referential agent. The cytotoxicity assay was performed after 72 h exposure of the cultured cells at the concentration ranging from 0.1 to 100 μg/ml of the tested agents. The SRB test measuring the cell proliferation inhibition in *in vitro* culture was applied.[30] The cells attached to the plastic were fixed with cold 50% TCA (trichloroacetic acid, Aldrich-Chemie, Germany) added on the top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (SRB, Sigma, Germany) dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 min. The unbound dye was removed by rinsing (four times) with 1% acetic acid, and the protein-bound dye was extracted with 10 mM unbuffered Tris base (tris (hydroxymethyl) aminomethane, POCh, Gliwice, Poland) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Uniskan II (Labsystems, Helsinki, Finland). The compounds were tested in triplicates per experiment. The experiments were repeated at least 3 times.

Preparation of Compounds. 2-Amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (I) A mixture of semicarbazide hydrochloride (Alfa Aesar) (0.01 mol) and STB (0.0075 mol) in methanol (50 ml) and pyridine (5 ml) was refluxed for 3 h. The reaction mixture was hot filtered. The resulted product was filtered off, washed with benzene and crystallized from methanol (30 ml). mp 328—330 °C. ^{1}H-NMR (500 MHz, $CDCl_3$), δ: 12.60 (s, 1H, C_2–OH), 10.55 (s, 1H, C_4–OH), 9.92 (s, 2H, NH_2), 7.58—7.57 (d, J=8.7 Hz, 1H, C_6–H), 6.43 (d, J=2.3 Hz, 1H, C_3–H), 6.37—6.35 (dd, J=8.5, 2.3 Hz, 1H, C_5–H). IR (KBr) cm^{-1}: 3176 (OH, NH), 1641 (C=N), 1620, 1602 (C=C), 1516, 1494, 1476, 1434, 1374, 1332, 1291, 1267, 1244, 1203 (C–OH), 1176, 1145, 1130, 991, 970, 856, 692 (C–S–C). EI-MS *m/z* (%): 210 (100), 153 (14), 150 (15), 122 (3), 121 (8), 108 (3), 97 (5), 94 (20), 81 (5), 75 (4), 69 (7), 66 (13), 39 (7). *Anal.* Calcd for $C_8H_7N_3O_2S$ (209.23): C, 45.92; H, 3.37; N, 20.08. Found: C, 45.79; H, 3.38; N, 20.01.

2-Hexylamino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (II) A mixture of 4-hexyl-3-thiosemicarbazide (Fluorochem) (0.01 mol) and STB (0.0075 mol) in methanol (50 ml) was refluxed for 3 h. The reaction mixture was hot filtered, and water (50 ml) was added to the filtrate. The precipitated product was filtered off, washed with water and crystallized from methanol (30 ml). mp 171—172 °C. ^{1}H-NMR (500 MHz, $CDCl_3$,) δ: 10.91 (s, 1H, C_2–OH), 9.14 (s, 1H, C_4–OH), 7.80 (s, 1H, NH), 7.53—7.51 (d, J=8.3 Hz, 1H, C_6–H), 6.37 (m, 1H, C_3–H), 6.35—6.34 (m, 1H, C_5–H), 3.42 (q, 2H, CH_2N), 1.50—1.47 (m, 2H, CH_2), 1.30—1.24 (m, 6H, CH_2), 0.88—0.85 (t, 3H, CH_3). IR (KBr) cm^{-1}: 3264, 3138 (OH, NH), 2925, 2855 (CH_2), 1627 (C=N, C=C), 1566, 1543, 1468, 1437, 1343, 1299, 1270, 1243, 1184 (C–OH), 1117, 1093, 1059 (N=C–S–C=N), 1014, 988, 939, 868, 836. EI-MS *m/z* (%): 293 (M^+, 39), 279 (8), 264 (11), 260 (9), 258 (30), 251 (18), 222 (17), 213 (10), 209 (30), 194 (10), 192 (30), 167 (25), 162 (13), 159 (12), 136 (14), 135 (20), 130 (10), 127 (24), 116 (24), 100 (17), 64 (100), 55 (21), 43 (58). *Anal.* Calcd for $C_{14}H_{19}N_3O_2S$ (293.38): C, 57.31; H, 6.53; N, 14.32. Found: C, 57.17; H, 6.50; N, 14.37.

2-Cyclohexylamino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (III), 2-(2,5-dichlorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (V), 2-(4-iodophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (VIII), 2-(5-chloro-2-methylphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (IX), 2-(4-ethoxycarbonylphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (XII) were described previously.[25]

2-(2,4-Dimethylphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (IV) A mixture of 4-(2,4-dimethylphenyl)-3-thiosemicarbazide (Alfa Aesar) (0.01 mol) and STB (0.0075 mol) in methanol (50 ml) was refluxed for 3 h. The reaction mixture was hot filtered, and the filtrate was concentrated to 20 ml. The precipitated product was filtered off, washed with water and crystallized from methanol (50 ml). mp 118—120 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.90 (s, 1H, C_2–OH), 9.92 (s, 1H, C_4–OH), 9.52 (s, 1H, NH), 7.71—7.69 (d, J=8.6 Hz, 1H, C_6–H), 7.67—7.65 (d, J=7.9 Hz, 1H, Ar′–H), 7.19—7.15 (m, 1H, Ar′–H), 7.06—6.96 (m, 1H, Ar–H′), 6.43 (d, J=2.4 Hz, 1H, C_3–H), 6.39—6.36 (dd, J=8.6, 2.4 Hz, 1H, C_5–H), 2.28—2.24 (m, 6H, CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 165.37 (C_{thia}-2), 160.11 (C-2), 155.87 (C-4), 154.87 (C_{thia}-5), 136.78 (C′), 133.31 (C′), 131.24 (2C′), 129.75 (C′), 128.51 (C-6), 127.07 (2C′), 122.07 (C′), 108.38 (C-1), 108.02 (C-5), 102.45 (C-3), 20.36 (CH_3), 17.75 (CH_3). IR (KBr) cm^{-1}: 3411, 3190 (OH, NH), 2918 (CH_2), 1624 (C=N), 1596 (C=C), 1530 1467, 1408, 1350, 1310, 1211 (C–OH), 1122, 984, 965, 874, 845, 802, 670 (C–S–C). EI-MS *m/z* (%): 313 (M^+, 100), 298 (12), 280 (8), 237 (6), 179 (11), 178 (96), 163 (20), 153 (11), 151 (17), 146 (21), 145 (58), 144 (22), 136 (8), 135 (8), 131 (11), 121 (24), 120 (25), 119 (25), 106 (14), 105 (8), 91 (11), 77 (12). *Anal.* Calcd for $C_{16}H_{15}N_3O_2S$ (313.38): C, 61.32; H, 4.82; N, 13.41. Found: C, 61.17; H, 4.84; N, 13.46.

2-(2,6-Dichlorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (VI) A mixture of 4-(2,6-dichlorophenyl)-3-thiosemicarbazide (Lancaster) (0.01 mol) and STB (0.0075 mol) in methanol (50 ml) was refluxed for 3 h. The reaction mixture was hot filtered and the filtrate was concentrated to dry. The precipitated product was dissolved in aqueous (1 : 1) methanol (50 ml) and filtrated off, washed with water and crystallized from methanol (30 ml). mp 136—138 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.73 (s, 1H, C_2–OH), 9.88 (s, 1H, C_4–OH), 7.72—7.70 (d, J=8.4 Hz, 1H, C_6–H), 7.59—7.57 (d, J=8.1 Hz, 2H, $C_{3',5'}$–H), 7.36—7.32 (t, J=8.1 Hz, 1H, $C_{4'}$–H), 6.43 (d, J=2.4 Hz, 1H, C_3–H), 6.38—6.35 (dd, J=8.6, 2.4 Hz, 1H, C_5–H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 165.37 (C_{thia}-2), 160.15 (C-2), 155.00 (C-4), 155.55 (C_{thia}-5), 135.02 (C′), 132.73 (C′), 128.98 (2C′), 128.60 (C-6), 128.30 (2C′), 108.54 (C-1), 108.05 (C-5), 102.41 (C-3). IR (KBr) cm^{-1}: 3366, 3158 (OH, NH), 1617 (C=N), 1598 (C=C), 1570, 1531, 1451, 1438, 1316, 1285, 1198 (C–OH), 1124, 1093 (C–Cl), 986, 965, 875, 845, 670 (C–S–C). EI-MS *m/z* (%): 354 (M^+, 11), 321 (7), 320 (40), 319 (18), 318 (100), 220 (4), 218 (5), 188 (3), 186 (9), 185 (12), 184 (13), 183 (28), 160 (6), 159 (8), 153 (10), 136 (6), 135 (70), 133 (6), 124 (5), 97 (4), 94 (7), 94 (74), 69 (5), 66 (4). *Anal.* Calcd for $C_{14}H_9Cl_2N_3O_2S$ (354.22): C, 47.47; H, 2.56; N, 11.86. Found: C, 47.23; H, 2.58; N, 12.01.

2-(4-Bromophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (VII) A mixture of 4-(4-bromophenyl)-3-thiosemicarbazide (Alfa Aesar) (0.01 mol) and STB (0.0075 mol) in methanol (50 ml) was refluxed for 3 h. The reaction mixture was hot filtered, and water (50 ml) was added to the filtrate. The precipitated product was filtered off and crystallized from methanol (50 ml). mp 236—237 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.62 (s, 1H, C_2–OH), 7.82—7.80 (d, J=8.6 Hz, 1H, C_6–H), 7.66—7.64 (d, J=8.9 Hz, 2H, $C_{3',5'}$–H), 7.52—7.50 (d, J=8.9 Hz, 2H, $C_{2',6'}$–H), 6.48 (d, J=2.3 Hz, 1H, C_3–H), 6.42—6.39 (dd, J=8.6, 2.2 Hz, 1H, C_5–H). IR (KBr) cm^{-1}: 3240 (OH, NH), 1613 (C=N), 1586 (C=C) 1564, 1532, 1484, 1401, 1322, 1263, 1210 (C–OH), 1183, 1132, 1105, 1075 (N=C–S–C=N), 1007, 985, 965, 849, 823, 668 (C–S–C). EI-MS *m/z* (%): 365 (99), 364 (M^+, 24), 363 (100), 334 (3), 302 (5), 284 (2), 230 (34), 228 (36), 227 (2), 214 (3), 213 (10), 196 (9), 195 (3), 168 (2), 167 (7), 157 (8), 155 (9), 153 (20), 159 (18), 149 (41), 142 (16), 136 (11), 135 (24), 122 (14), 121 (10), 108 (11), 94 (32), 91 (11), 90 (12), 76 (13), 75 (13), 69 (12), 66 (20), 65 (10), 63 (12), 52 (12). *Anal.* Calcd for $C_{14}H_{10}BrN_3O_2S$ (364.22): C, 46.17; H, 2.77; N, 11.54. Found: C, 45.98; H, 2.49; N, 11.48.

2-(3-Chloro-4-methylphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (X) A mixture of 4-(3-chloro-4-methylphenyl)-3-thiosemicarbazide (Lancaster) (0.01 mol) and STB (0.0075 mol) in methanol (50 ml) was refluxed for 3 h. The reaction mixture was hot filtered, and water (40 ml) was added to the filtrate. The precipitated product was filtered off and crystallized from methanol (50 ml). mp 247—248 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.19 (s, 1H, C_2–OH), 10.51 (s, 1H, C_4–OH), 9.77 (broad band, 1H, NH), 7.94—7.93 (d, J=2.4 Hz, 1H, $C_{2'}$–H), 7.83—7.80 (d, J=8.6 Hz, 1H, C_6–H), 7.40—7.37 (dd, J=8.7, 2.2 Hz, 1H, $C_{6'}$–H), 7.31—7.30 (d, J=8.8 Hz, 1H, $C_{5'}$–H), 6.47 (d, J=2.4 Hz, 1H, C_3–H), 6.44—6.41 (dd, J=8.6, 2.4 Hz, 1H, C_5–H), 2.29 (s, 1H, CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 163.30 (C_{thia}-2), 160.65 (C-2), 155.95 (C-4), 155.24 (C_{thia}-5), 139.86 (C′), 133.41 (C′), 131.45 (C′), 128.59 (C-6), 128.14 (C′), 117.41 (C′), 116.23 (C′), 108.31 (C-1), 108.20 (C-5), 102.53 (C-3), 18.83 (CH_3). IR (KBr) cm^{-1}: 3247 (OH, NH), 1612 (C=N, C=C), 1558, 1528, 1495, 1452, 1322, 1223 (C–OH), 1179, 1113 (C–Cl), 1044 (N=C–S–C=N), 987, 968, 932, 909, 669 (C–S–C). EI-MS *m/z* (%): 333 (M^+, 100), 302 (8), 200 (9),

199 (5), 198 (23), 197 (8), 184 (3), 168 (3), 167 (9), 166 (5), 163 (51), 153 (14), 150 (9), 149 (7), 141 (6), 140 (6), 136 (10), 135 (13), 131 (6), 125 (7), 121 (7), 108 (5), 97 (5), 94 (20), 89 (9), 77 (10), 69 (7), 66 (11), 63 (7), 52 (7), 39 (10). *Anal.* Calcd for $C_{15}H_{12}ClN_3O_2S$ (333.79): C, 53.97; H, 3.62; N, 12.59. Found C, 53.72; H, 3.64; N, 12.54.

2-(4-Methoxyphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (XI) A mixture of 4-(4-methoxyphenyl)-3-thiosemicarbazide (Aldrich) (0.01 mol) and STB (0.0075 mol) in methanol (50 ml) was refluxed for 3 h. The reaction mixture was hot filtered, and water (40 ml) was added to the filtrate. The precipitated product was filtered off and crystallized from methanol (50 ml). mp 198—200 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.10 (broad band, 1H, C_2–OH), 10.26 (s, 1H, C_4–OH), 9.78 (broad band, 1H, NH), 7.75—7.73 (d, J=8.6 Hz, 1H, C_6–H), 7.55—7.53 (m, J=9.2, 3.5 Hz, 2H, Ar′–H), 6.96—6.93 (m, J=9.2, 3.5 Hz, 2H, Ar′–H), 6.44 (d, J=2.4 Hz, 1H, C_3–H), 6.42—6.39 (dd, J=8.6, 2.4 Hz, 1H, C_5–H), 3.74 (s, 3H, CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 164.27 (C_{thia}-2), 160.48 (C-2), 155.95 (C-4), 154.76 (C_{thia}-5), 154.44 (C′), 133.94 (C′), 128.52 (C-6), 119.69 (2C′), 114.35 (2C′), 108.18 (C-1), 108.02 (C-5), 102.46 (C-3), 55.27 (CH_3). IR (KBr) cm^{-1}: 3392, 3206 (OH, NH), 2955, 2837 (CH_3), 1626 (C=N), 1581 (C=C), 1510, 1455, 1411, 1329, 1305, 1253, 1221 (C–OH), 1187, 1113, 1032 (N=C–S–C=N), 987, 967, 821, 813, 679 (C–S–C). EI-MS m/z (%): 315 (M^+, 100), 314 (5), 302 (5), 301 (5), 300 (30), 180 (6), 167 (4), 165 (20), 153 (7), 150 (6), 148 (8), 136 (3), 135 (19), 133 (8), 122 (11), 121 (5), 108 (7), 107 (4), 95 (5), 94 (11), 66 (7), 64 (4), 52 (6), 39 (6). *Anal.* Calcd for $C_{15}H_{13}N_3O_3S$ (315.35): C, 57.13; H, 4.16; N, 13.33. Found: C, 56.92; H, 4.18; N, 13.37.

2-(Benzylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (XIII) A mixture of 4-benzyl-3-thiosemicarbazide (Lancaster) (0.01 mol) and STB (0.0075 mol) in methanol (50 ml) was refluxed for 3 h. The reaction mixture was hot filtered, and water (50 ml) was added to the filtrate. The precipitated product was filtered off and crystallized twice from methanol (30 ml). mp 230—231 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.10 (broad band, 1H, C_2–OH), 10.26 (s, 1H, C_4–OH), 10.03 (s, 1H, NH), 7.78—7.76 (d, J=8.6 Hz, 1H, C_6–H), 7.55—7.53 (m, 4H, Ar′–H), 6.96—6.93 (m, 1H, Ar′–H), 6.46 (d, J=2.4 Hz, 1H, C_3–H), 6.42—6.39 (dd, J=8.6, 2.4 Hz, 1H, C_5–H), 3.27 (s, 2H, CH_2). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 163.75 (C_{thia}-2), 160.46 (C-2), 155.91 (C-4), 154.73 (C_{thia}-5), 138.23 (C-4′), 130.86 (C-1′), 129.45 (2C-2′,6′), 128.55 (C-6), 117.71 (2C-3′,5′), 108.17 (C-1), 108.10 (C-5), 102.46 (C-3), 20.32 (CH_2). IR (KBr) cm^{-1}: 3191 (OH, NH), 1618 (C=N), 1575 (C=C), 1515, 1455, 1317, 1220 (C–OH), 1181, 1112, 1018, 987, 966, 878, 673 (C–S–C). EI-MS m/z (%): 299 (M^+, 100), 298 (12), 270 (3), 167 (4), 165 (5), 164 (37), 163 (17), 153 (11), 150 (13), 137 (4), 136 (5), 135 (11), 132 (8), 131 (13), 121 (7), 107 (5), 106 (10), 105 (6), 104 (4), 97 (5), 94 (19), 91 (15), 79 (8), 78 (5), 77 (9), 69 (6), 66 (12), 65 (13), 39 (10). *Anal.* Calcd for $C_{15}H_{13}N_3O_2S$ (299.35): C, 60.18; H, 4.38; N, 14.04. Found: C, 59.17; H, 4.36; N, 13.99.

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