In vitro biological evaluation of novel N-substituted 2,4-dihydroxythiobenzamides

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As continuation of the studies on the synthesis and evaluation of biologically active compounds functionalized by 2,4-dihydroxyphenyl substituent, a series of N-modified thiobenzamides have been prepared. They have been obtained by the reaction of the corresponding amines with the electrophilic thioacylating agent. The compounds have been subjected to in vitro antifungal activity screening against phytopathogenic fungi and antiproliferative potency against human cancer cell lines. N-(2,1,3-Benzothiadiazol-5-yl)-2,4-dihydroxybenzothioamide exhibits the highest antifungal activity, especially against R. solani and P. cactorum. 2,4-Dihydroxy-N’-(3-methyl-1,3-benzothiazol-2(3H)-ilidene)benzothiohydrazide shows high antiproliferative potency against the SW707 cells at IC50 = 2 µM, similar to that of cisplatin. To explain differences in the activity of the compounds, their in silico properties have been estimated.

Keywords: 2,4-Dihydroxythiobenzamides, synthesis, antifungal activity, antiproliferative potency, phytopathogenic fungi

Benzamide derivatives, including their sulfur analogs thiobenzamides, are a group of compounds exhibiting a wide spectrum of biological potency, especially antimicrobial one. Thiobenzamides and other carbothioamides are mainly known as antimycobacterial agents1-4. For example, strong inhibitory activity for 3'-fluoro- and 4'-fluorothiobenzanilides was demonstrated against Mycobacterium tuberculosis, M. kansasii, M. avium and M. fortuitum. Some compounds from a series of 4-alkylthiopyridine-2-carbothioamides showed antimycobacterial activity comparable to that of isoniazide3. Simultaneously, much attention was paid to the structure-antituberculosis activity elucidation3,4.

The other carbothioamides are known antymycotic agents. They show antifungal activity against some strains of yeast-like3,6, moulds, deramatophytes and a number of phytopathogenic fungi7,9. Series of 2-alkylthiopyridine-4-carbothioamides, 4-alkylthiopyridine-2-carbothioamides and 2-benzylthiopyridine-4-carbothioamides were described as antifungals of substantial potency3,7,9. Antibacterial activity for 3,4,4'-trichlorothiobenzanilide was described against Staphylococcus aureus10. This group of substances also exhibits herbicidal and insecticidal activity10-12. Quinolizidine derivatives of thiobenzamide were described as influenza fusion inhibitors11,12; quinoline-3-carbothioamides as novel immunomodulating agents13, another heterocyclic derivative of carbothioamide is under preclinical evaluation as an antidepressant14.

Many heterocyclic derivatives functionalized by 2,4-dihydroxyphenyl containing the linear >NC(=S)–moiety, thioamides, amidrazones and hydrazides or incorporated in the heterocyclic ring, 1,3,4-thiadiazoles and benzothiazoles show strong antifungal and antiproliferative effects5-17. Some of them exhibit significant mycelium growth inhibition of phytopathogenic fungi18,19. In vivo this effect was confirmed against Erisiphe graminis18.

To extend our research in this field, a set of new compounds with benzenediol functionality was prepared. The paper presents in vitro antifungal properties of new N-substituted 2,4-dihydroxythiobenzamides against five phytopathogenic fungi. Some compounds were evaluated for their antiproliferative potency against human cancer cell lines. To explain differences in the activity, certain properties of compounds in silico were determined.
Results and Discussion

N-substituted 2,4-dihydroxythiobenzamides (1-6) were prepared from corresponding amines (1a-5a) or hydrazine (6a) and sulfonlules[(2,4-dihydroxyphenyl) methanethione] (STB), as the electrophile reagent. The synthetic pathway and structures of compounds are presented in Figure 1. STB was obtained according to the previously described procedure. Some analytical data of the compounds are summarized in Table I. They are in agreement with the proposed structures. The peak of molecular ion (M⁺) is visible in the EI-MS spectra of all compounds. They are however, of different intensity. 153 m/z band corresponding to [(HO)₂C₆H₅(C=S)]⁺ is characteristic of all analogs. M⁺-SH peak is also registered for most of the derivatives. This is a characteristic fragmentation of other 2,4-dihydroxythiobenzamide derivatives. The labile NH and OH protons are invisible in ¹H NMR in the presence of D₂O. The ¹³C NMR spectra of the compounds show a low field signal at δ 193 which is attributed to carbon atom of C=S group. Purity of the compounds was monitored by the reversed-phase HPLC (CP-18, methanol-water mobile phase). The log k values for the selected system are collected in Table I.

The compounds were in vitro evaluated against five strains of phytopathogenic fungi at two different concentrations. The results are given in Table II.
Procymidone and carbendazim were used as the reference systems. It follows from Table II that the activity of compounds is varied. The highest potency has been found for compounds 5 and 6. The first one at the concentration of 200 μg/mL showed fungistatic action at the level 61-80% against *R. solani*, *F. culmorum*, *P. cactorum* and at the concentration of 20 μg/mL caused inhibition of mycelium growth at 41-60% of *R. solani* and *P. cactorum*. Derivative 6 showed a similar effect but at higher concentration only. *R. solani* and *A. alternata* seem to be particularly susceptible to the tested set of compounds. Compound 1 with the N-phenyl substituent was completely inactive while other analogs showed low activities. Taking into account the structure of the compounds, the derivative with 2,1,3-benzothiadiazole moiety was indicated as the most active. Comparing the activity of new compounds with the previously described analogs, it was found that their activity is lower than that of most active compound, which caused 81-100% mycelium growth inhibition at the concentration of 200 μg/mL18.

The antiproliferative activity of the two compounds has been evaluated against four human cancer cells: T47D (breast cancer), SW707 (rectal adenocarcinoma), and A549 (non-small cell lung carcinoma) and was expressed as IC50 (μM) (Table III). Both compounds showed substantial antiproliferative potency against all studied cells. The activity of 2,4-dihydroxy-N’-(3-methyl-1,3-benzothiazol-2(3H)-ilidene)benzothiohydrazide (6) toward SW707 cells was better than that of cisplatin, when studied comparatively. Its activity was found to be similar to that exhibited by the other best compounds with benzenediol moiety22.

To explain the differences in the activities of the compounds, some physico-chemical properties and molecular descriptors of molecules were estimated *in silico*. They are collected in Table IV. The data show that inactive compound 1 possesses the considerably low distribution coefficient Clog P and the largest number of rotatable bonds. It can be assumed that low lipophilicity and a flexible molecule are not conducive to antifungal activities against the studied strains. It follows from Table IV that all obtained compounds meet the Lipinski’s rule of five: their MW ≤ 500, Clog P ≤ 5, HBD ≤ 5 and HBA ≤ 10 (Table IV)23,24. Therefore it can be assumed that the

### Table II — Antifungal activity of compounds 1-6 against phytopathogenic fungi

<table>
<thead>
<tr>
<th>Entry</th>
<th>A. alternata</th>
<th>B. cinerea</th>
<th>R. solani</th>
<th>F. culmorum</th>
<th>P. cactorum</th>
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<tbody>
<tr>
<td></td>
<td>Estimation of mycelium growth inhibition* / (mg/L)</td>
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<td></td>
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<tr>
<td>1</td>
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<td>-†</td>
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<tr>
<td>Procymidon</td>
<td>2 2 3 3</td>
<td>-†</td>
<td>-†</td>
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<td>-†</td>
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</table>

* The results are given in the five-degree scale determining in percent of mycelium growth inhibition compared with the control: 0 = 0-20%; 1 = 21-40%; 2 = 41-60%; 3 = 61-80%; 4 = 81-100%.
† Study was not performed

### Table III — Antiproliferative activity of some compounds against human cancer cell lines

<table>
<thead>
<tr>
<th>Entry</th>
<th>HCV29T</th>
<th>A 549</th>
<th>T 47D</th>
<th>SW 707</th>
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<tr>
<td>2</td>
<td>43.14 ± 5.64</td>
<td>58.69 ± 11.12</td>
<td>63.55 ± 5.10</td>
<td>23.79 ± 4.05</td>
</tr>
<tr>
<td>6</td>
<td>12.71 ± 3.38</td>
<td>12.73 ± 3.38</td>
<td>16.47 ± 1.2</td>
<td>1.84 ± 3.91</td>
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<tr>
<td>Cisplatin</td>
<td>3.10 ± 0.96</td>
<td>6.77 ± 5.37</td>
<td>6.47 ± 2.27</td>
<td>10.43 ± 4.97</td>
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</table>

* IC50: indicates the compound concentration that inhibits the proliferation rate of tumor cells by 50% as compared to the control untreated cells. The values are means ± SD of nine independent experiments
Experimental Section

Melting points (m.p.) were determined using a Büchi B-540 (Flawil, Switzerland) melting point apparatus. The elemental analysis (CHN) was performed on Perkin-Elmer 2400. The IR spectra were measured with a Perkin-Elmer FT-IR 1725X spectrophotometer (in KBr) or a Varian 670-IR FT-IR spectrometer (ATR) in the range of 600-4000 cm⁻¹. The NMR spectra (1D NMR) were recorded in DMSO-d₆ using a Varian Mercury 400, Bruker DRX 500 (Bruker Daltoncs, Inc. Billerica, MA, USA) or Tesla BS 567A (100 MHz). Chemical shifts (δ, ppm) were described in relation to tetramethylsilane (TMS) and coupling constants (J) were expressed in Hz. The MS spectra (EI, 70 eV) were recorded using the apparatus AMD-604.

The purity of the compounds was examined by HPLC Knauer (Berlin, Germany) with a dual pump, a 20 μL simple injection valve and a UV-Visible detector (275 and 330 nm). The Hypersil Gold C18 (1.9 μm, 100 × 2.1 mm) column was used as the stationary phase. The mobile phase included different contents of MeOH and acetate buffer (pH 4, 20 nM) as the aqueous phase. The flow rate was 0.4 mL/min at RT. The retention time of an un-retained solute (t₀) was determined by KCl. The log k values for 80% or 65% of MeOH (v/v) in the mobile phase are presented. They were calculated as log k = log(tR - t₀)/t₀, where:

Table IV — Molecular descriptors in silico of N-substituted 2,4-dihydroxythiobenzamides

<table>
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<tr>
<th>Entry</th>
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<th>HBA</th>
<th>logP</th>
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<th>RB</th>
<th>tPSA (Å²)</th>
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</table>

* - Clog P - the octanol-water partition coefficients; HBD - a number of H-bond donors; HBA - a number of H-bond acceptors; RBC - a number of rotatable bonds; NR - a number of rings; tPSA - the polar surface area; MR - the molar refractivity

The purity of the compounds was examined by HPLC Knauer (Berlin, Germany) with a dual pump, a 20 μL simple injection valve and a UV-Visible detector (275 and 330 nm). The Hypersil Gold C18 (1.9 μm, 100 × 2.1 mm) column was used as the stationary phase. The mobile phase included different contents of MeOH and acetate buffer (pH 4, 20 nM) as the aqueous phase. The flow rate was 0.4 mL/min at RT. The retention time of an un-retained solute (t₀) was determined by KCl. The log k values for 80% or 65% of MeOH (v/v) in the mobile phase are presented. They were calculated as log k = log(tR - t₀)/t₀, where:

General procedure for the synthesis of compounds 1–6

A mixture of the corresponding aryl- (1a), heterocyclic amine (2a-5a) or hydrazone (6a) (0.01 mmol) and electrophile STB (0.01 mmol) in MeOH (50 mL) was treated to reflux for 3 h. The hot mixture was filtered via a Büchner funnel. The filtrate was concentrated to small volume and the formed solid was filtered off (compounds 1, 3, 4), the filtrate was left at RT (24 h) and filtered (5). In the case of compound 6 water (100 mL) was added to the filtrate. The compounds were purified by recrystallization from MeOH (50 mL) (compounds 1, 4) or from MeOH/H₂O solution (50 mL) (compounds 2, 3, 5, 6).

Diethyl 4-(2,4-dihydroxyphenylthioamido)benzyl phosphonate, 1: ¹H NMR (400 MHz, DMSO-d₆): δ 11.52 (s, 1 H, HO-C(2), 11.25 (s, 1 H, HO-C(4), 10.04 (s, 1 H, NH), 7.84 (d, 1 H, J = 8.9 Hz, H-C(6), 7.66 (d, 2 H, H-C (Ar), 7.30 (d, 2 H, J = 6.9 Hz, H-C (Ar), 6.35 (s, 1 H, H-C(3)), 6.33 (m, 1 H, H-C(5)), 3.96 (q, J = 7.3 Hz, 4 H, CH₂), 3.24 (d, J = 21.5 Hz, 2 H, CH₂), 1.19 (t, J = 7.3 Hz, 6 H, CH₃); IR (KBr): 3412 (OH, NH), 3168 (OH), 2983 (CH), 2920 (CH), 1624 (C=C), 1584 (C=C), 1512 (C=C), 1467 (C-N), 1395, 1346, 1332 (N-H), 1291, 1216 (C-OH), 1191, 1162, 1121 (C=S), 1016, 987, 859, 810, 764 cm⁻¹; MS: m/z (%) 395 (M+, 3), 362 (M+-SH, 57), 334 (9), 288 (20), 243 (16), 225 (18), 214 (6), 186 (5), 168 (3), 153 (41), 137 (7), 124 (4), 109 (15), 106 (100), 97 (10), 90 (21), 81 (22), 77 (15), 69 (10), 65 (5), 62 (51), 47 (46), 45 (32), 39 (9), 36 (6).
N-(4-(5-Chloro-1,3-dioxoisololin-2-yl)-3-methylphenyl)-2,4-dihydroxybenzothioamide, 3: $^1$H NMR (500 MHz, DMSO-$d_6$): δ 11.62 (s, 1 H, HO-C(2)), 11.21 (s, 1 H, HO-C(4)), 10.07 (s, 1 H, NH), 8.08 (m, 1 H, H-C(Ar)), 7.99 (m, 2 H, H-C(Ar)), 7.86 (d, 1 H, J = 7.9 Hz, H-C(6)), 7.76 (s, 1 H, H-C(3)), 7.39 (d, J = 8.4 and 2.1 Hz, 1 H, H-C(Ar)), 6.38-6.30 (m, 2 H, H-C(3,5)), 2.14 (s, 3 H, CH$_3$); $^{13}$C NMR (125 MHz, DMSO): δ 193.8 (C=S), 166.3 (C=O), 165.4 (C=O), 161.9, 157.2, 140.4, 139.8, 137.9, 136.8, 134.6, 133.7, 133.4, 130.2, 129.5, 128.2, 126.5, 125.4, 123.7, 122.9, 107.8, 102.9, 17.8 (CH$_3$); IR (KBr): 3452 (OH, NH), 1713 (C=O), 1618 (C=C), 1585 (N-H), 1384, 1181 (C-OH), 1119 (C=S), 1020, 841, 810, 690 cm$^{-1}$; MS: m/z (%) 438 (M'$_2$, 36), 405 (M'-SH, 100), 303 (10), 286 (26), 268 (8), 242 (4), 219 (5), 153 (12), 136 (4), 110 (3), 97 (2), 77 (3), 53 (2).

Methyl 3-(2,4-dihydroxyphenylthioamido)thiophene-2-carboxylate, 4: $^1$H NMR (100 MHz, DMSO-$d_6$): δ 12.73 (s, 1 H, HO-C(2)) (an exchangeable in D$_2$O), 11.55 (s, 1 H, HO-C(4)) (an exchangeable in D$_2$O), 10.25 (s, 1 H, NH) (an exchangeable in D$_2$O), 8.71 (d, J = 5.4 Hz, 1 H, H-C(tiof)), 8.23 (d, J = 9.6 Hz, 1 H, H-C(6)), 7.90 (d, J = 5.4 Hz, 1 H, H-C(thio)), 6.43 (m, 1 H, H-C(3)), 6.36 (d, J = 2.3 Hz, 1 H, H-C(5)), 3.85 (s, 3 H, CH$_3$); $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 193.0 (C=S), 162.4 (CO), 162.3 (Cthio), 156.0 (Cthio), 144.2 (Cthio), 136.1 (Cthio), 130.9, 124.7, 117.6, 115.8, 108.2, 102.0, 52.1 (CH$_3$); IR (KBr): 3420 (OH, NH), 3277 (OH), 3219 (OH), 3122 (OH), 3096 (C=Ar-H), 2952 (CH), 2830 (CH), 1677 (C=O), 1627 (C=C), 1574 (C=C), 1516 (C=C), 1469 (C=N), 1444 (C=H), 1419, 1393, 1346 (N-H), 1267 (C=N), 1125 (C=S), 1104, 1093, 986, 967, 941, 926, 889, 854, 845, 800, 774, 752, 717 cm$^{-1}$; MS: m/z (%) 309 (M', 91), 276 (M'-SH, 51), 261 (3), 250 (100), 244 (4), 234 (19), 219 (8), 208 (13), 192 (5), 185 (5), 168 (3), 157 (27), 153 (34), 137 (12), 125 (19), 115 (4), 108 (5), 97 (7), 81 (3), 69 (10), 53 (4), 45 (7), 39 (8).

N-(2,1,3-Benzothiadiazol-5-yl)-2,4-dihydroxybenzothioamide, 5: $^1$H NMR (500 MHz, DMSO-$d_6$): δ 11.81 (s, 1 H, HO-C(2)), 10.71 (s, 1 H, HO-C(4), 9.71 (s, 1 H, NH), 7.84 (d, J = 9.04 Hz, 1 H, H-C(6)), 7.70 (m, 2 H, H-C(benzothiadiazole), 6.72 (m, 1 H, H-C(benzothiadiazole), 6.38 (dd, J = 9.4 and 2.4 Hz, 1 H, H-C(5)), 6.33 (d, J = 2.40 Hz, 1 H, H-C(3)); IR (ATR): 3265 (NH, OH), 2942 (C-H), 1618 (C=N), 1588 (C=C), 1554 (C=C), 1469 (C=N), 1438, 1344 (N-H), 1262, 1207 (C=OH), 1153, 1120 (C=S), 1052, 983, 849, 810, 740, 690 cm$^{-1}$; MS: m/z (%) 303 (M'$^+$, 100), 285 (3), 273 (100), 244 (3), 232 (2), 168 (12), 153 (93), 135 (10), 116 (3), 97 (7), 81 (3), 69 (5), 53 (3), 45 (3), 39 (3).

2,4-Dihydroxy-N'-(3-methyl-1,3-benzothiazol-2(3H)-ilidene)benzothiohydrazide, 6: $^1$H NMR (100 MHz, DMSO-$d_6$): δ 11.05 (s, 1 H, HO-C(2)) (exchangeable in D$_2$O), 10.68 (s, 1 H, HO-C(4)) (an exchangeable in D$_2$O), 10.00 (s, 1 H, NH (exchangeable in D$_2$O), 7.95 (d, J = 8.7 Hz, 1 H, H-C(6)), 7.34-7.52 (m, 2 H, H-C(Ar)), 7.40-7.35 (m, 2 H, H-C(Ar)), 6.50 (m, 1 H, H-C(3)), 6.35 (m, 1 H, H-C(5)), 3.64 (s, 3 H, CH$_3$); $^{13}$C NMR (125 MHz, DMSO-$d_6$): δ 187.0 (C=S), 163.0 (C-N), 161.0, 156.8, 140.9, 129.5, 127.6, 126.5, 125.0, 122.9, 122.3, 121.6, 108.2, 102.2, 32.9 (CH$_3$); IR (KBr): 3224 (OH), 2931 (CH), 1626 (C=C), 1601 (C=O), 1476, 1419, 1332, 1250, 1235 (C=O), 1165, 1130 (C=S), 1024, 982, 959, 866, 843, 801, 743, 714 cm$^{-1}$; MS: m/z (%): 331 (M'$^+$, 40), 326 (4), 299 (29), 282 (5), 270 (2), 266 (6), 208 (7), 181 (100), 167 (5), 163 (25), 153 (7), 150 (33), 148 (37), 136 (55), 122 (13), 109 (27), 104 (14), 91 (2), 77 (10), 69 (10), 63 (8), 57 (7), 45 (9), 41 (4), 39 (7).

**Computational methods**

The Clog P values and molar refractivity MR were calculated using the ChemDraw Ultra 10.0 according to the fragmentation method introduced by Crippen$^{25,26}$. The tPSA were calculated by Virtual Computational Chemistry Laboratory$^{27}$. The polar surface area (tPSA) was calculated by the atom-based method$^{28}$.

**Biological assays**

**Antifungal activity**

The test in vitro estimating inhibition of mycelium growth in the agar culture medium caused by the compound under investigation was performed. The five strains of phytopathogenic fungi: Alternaria alternata, Botrytis cinerea, Rhizoctonia solani, Fusarium culmorum and Phytophthora cactorum were used. The solutions (3% suspensions) were prepared with the concentration making it possible to obtain 200 and 20 μg/mL of the studied substance after dilution with the agar culture medium (PDA). There
were used in Petri scale pans into which the agar culture medium and the studied substance were poured. When the culture medium sets, the infectious material of the tested fungus in the form of agar disc overgrown with mycelium is placed at three sites of its surface. After 3-5 days (temperature 22 (±1)°C) depending on the mycelium culture, the linear growth of the mycelium is measured. The compound action was determined from the percentage of mycelium growth inhibition compared with the control using the equation: $J = \frac{(C-T)}{C} \times 100\%$, where: $J$ – percentage of mycelium growth inhibition; $C$ – zone of mycelium growth in the control combination (mm); $T$ – zone of the mycelium growth in the combination with the compound (mm). Carbendazime (Sarfun 500 SC, Organica, Chemical Comp. S.A., Nova Sarzyna, Poland) and procymidone (Sumilex 500 SC, Sumitomo Chemical Comp. Ltd., Japan) were used as standards. The results are given in the five-degree scale determining the percentage of mycelium growth inhibition compared with the control (Table II). Biological studies were carried out in the Institute of Industrial Organic Chemistry in Warsaw with the SPR/BFF/01/b procedures (certificate GLP-OECD – 1997).

**Antiproliferative assay**

The following established in vitro human cell lines were used in this study: HCV29T (bladder cancer) from the Fibiger Institute, Copenhagen, Denmark, T47D (breast cancer), SW707 (rectal adenocarcinoma), and A549 (non-small cell lung carcinoma) from the American Type Culture Collection (Rockville, Maryland, USA). Twenty-four hours before the addition of the tested agents, the cells were plated in 96-well plates (Sarstedt Inc, Newton, NC, USA) at a density of 10^4 cells/well. All cell lines were maintained in the opti-MEM medium supplement with 2 mM glutamine (Gibco), streptomycin (50 µg/mL), penicillin (50 U/mL) (Polfa, Tarchomin), and 5% fetal calf serum (Gibco). The cells were incubated at 37°C in a humid atmosphere saturated with 5% CO₂. The solutions of compounds (1 mg/mL) were prepared extempore by dissolving the substance in 100 µL of DMSO followed by the addition of 900 µL of tissue culture medium. Afterwards, the compounds were diluted in the culture medium to the final concentrations ranging from 0.1 to 100 µg/mL. The solvent (DMSO) used at the highest concentration in the test did not reveal any cytotoxic activity. Cisplatin was used as a test reference agent. The cytotoxicity assay was performed after 72 h exposure of the cultured cells at the concentrations of tested agents ranging from 0.1 to 100 µg/mL. The SRB test was used to measure inhibition of cell proliferation in vitro (28). The cells attached to the plastic were fixed with cold 50% TCA (trichloroacetic acid, Sigma-Aldrich Chemie GmbH) added on the top of the culture medium in each well. The plates were incubated at 4°C for 1 h and then washed 5 times with tap water.

The background optical density was measured in the wells filled with the medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (SRB, Sigma-Aldrich Chemie GmbH) dissolved in 1% acetic acid (POCh) for 30 min^29. The unbound dye was removed by rinsing (4 times) with 1% acetic acid, and the protein-bound dye was extracted with 10 mM unbuffered Tris base (tris(hydroxymethyl) aminomethane, POCh) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Uniskan II (Labsystems). The compounds were tested in triplicate for each experiment, and the experiments were repeated at least 3 times.

**Conclusions**

Using the commercially available amines in the reaction with STB the novel N-substituted-2,4- dihydroxythiobenzamide derivatives were synthesised. The compounds caused mycelium growth inhibition of four phytopathogenic strains of fungi. They were also characterized by antiproliferative potency towards human cancer cell lines. 2,4-Dihydroxy-N’-(3-methyl-1,3-benzothiazol-2(3H)-ilidene)benzothiohydrazide showed antiproliferative potency toward SW707 at IC₅₀ = 2 µM and it seems to be an interesting compound as a potential anticancer agent.

**Declaration of interest**

The authors declare no conflict of interest.

**References**


27 http://www.vcclab.org
