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Synthesis, antiproliferative and antifungal activities of some 2-(2,4-dihydroxyphenyl)-4*H*-3,1-benzothiazines

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Abstract—A new method for the synthesis of 4*H*-3,1-benzothiazine skeleton is described. The compounds were obtained by the reaction of sulfinylbis(2,4-dihydroxythiobenzoyl) with *o*-substituted anilines bearing an activated methylene group (–CH_2OH, –$CH_2NR_1R_2$), *o*-aminobenzanilides or 2-aminobenzophenones. The reaction proceeded through thiobenzanilide intermediates, which were converted to the 4*H*-3,1-benzothiazine fused ring by an endocyclization process. The compounds were tested for their antiproliferative properties against the cells of a human breast cancer T47D line. The activity of some compounds was comparable to that of cisplatin, studied as a control. A strong antifungal effect against the strains of moulds, yeasts and dermatophytes was also found.

1. Introduction

The 4*H*-3,1-benzothiazine nucleus is a system that has been relatively unexplored with respect to both its synthesis and biological activity. 3,1-Benzothiazines are usually obtained by fusion of *o*-aminobenzyl chloride hydrochloride with different thiocarboamides, benzopyran-2-thiones or thiourea.[1] The reaction of *N*-phenylmethylketenimines with thiobenzophenones, according to 1,4-cycloaddition modes, also gives corresponding 4*H*-3,1-benzothiazines.[2] By treatment of [2-(*N*-acylamino)phenyl]alkanols with Lawesson's reagent, 2,4,4-trisubstituted derivatives and 4*H*-3,1-benzothiazin-2-ones were prepared in low yield, together with the linear sulfur or cyclic oxygen analogues.[3]

Besson and co-workers synthesized 4-oxoderivatives via *N*-arylimino-1,2,3-dithiazoles.[4,5] 4*H*-3,1-Benzothiazin-2-thione derivatives were obtained by the intramolecular heteroconjugate addition of carbodiimides or isothiocyanates bearing one *o*-substituted α,β-unsaturated carbonyl fragment and promoted by CS_2/TBAF.[6] The acid-catalyzed intramolecular conjugate addition of a thiourea to an α,β-unsaturated carbonyl intermediate product prepared on the solid phase is complementary to this methodology. In this way, 2-aminoderivatives were obtained.[7] 4-Imine derivatives have been synthesized from *N*-(2-cyanophenyl)benzimidoyl chloride and reagents containing a thioamide moiety.[8]

There is little information about the biological activity of 4*H*-3,1-benzothiazines.[5] However, other isomeric benzothiazines show a wide spectrum of pharmacological properties. They act as activators of ATP-sensitive potassium channels T-477,[9] as Ca^{2+} and Na^+ channel blockers,[10] showing anti-inflammatory,[11] antihypertensive,[12] antibacterial,[13] fungicidal[14] and antiproliferative[15] activities.

As part of a continuing programme in the development of synthetic routes to a new group of biologically active heterocyclic compounds possessing the –N=Ć–S– linkage, promoted by the 2,4-dihydroxyphenyl moiety, a method for the preparation of 2-(2,4-dihydroxyphenyl)-4*H*-3,1-benzothiazines has been elaborated. Derivatives were tested for their antiproliferative activity under in vitro conditions against the cells of human breast cancer T47D line. Cisplatin was used as the reference system. Antifungal properties were evaluated against different strains of moulds, yeasts and dermatophytes.

2. Results and discussion

Earlier studies on the reaction of sulfinylbis(2,4-dihydroxythiobenzoyl) (STB) with N^3-substituted amidraz-

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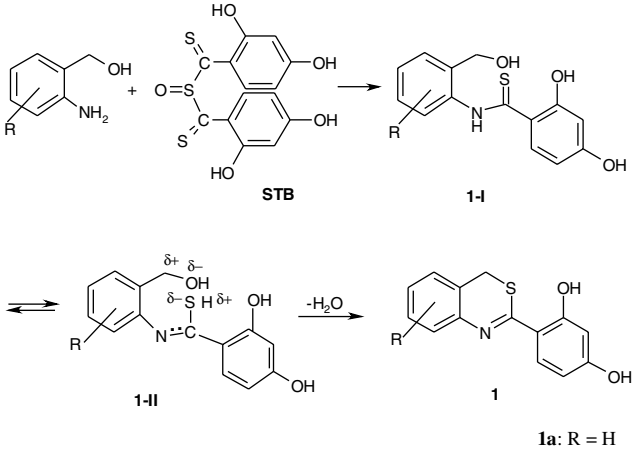
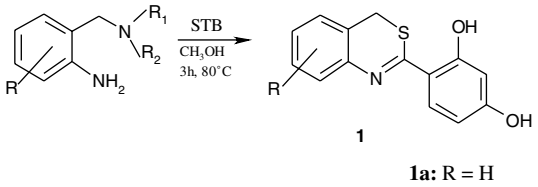
ones gave the linear product, N^1-thioacyl derivatives, and the cyclic one, 2,5-disubstituted 1,3,4-thiadiazoles.[16] This showed that the electrophilic reagent elaborated by our team can also perform the function of an endogenous cyclizing reagent. These properties were confirmed in the reactions with 4-substituted 3-thiosemicarbazides, hydrazides and carbazates, where a 1,3,4-thiadiazole ring was also obtained.[17] Therefore, we attempted to obtain another heterocyclic system—the 4*H*-3,1-benzothiazine ring.

At first we focused on *ortho*-substituted anilines bearing activated methylene groups such as $-CH_2OH$ and $-CH_2NR_1R_2$. From these compounds and STB, 2-(2,4-dihydroxyphenyl)-4*H*-3,1-benzothiazines were obtained (Schemes 1 and 2). The reaction of STB (as an electrophilic reagent) with substituted amines first gives arylthioamides. The transition product formed (**1-I**) then undergoes an intramolecular rearrangement to the imido-thiol form (**1-II**), characteristic of carbothioamides.[18] Interaction of the –OH group with thiol leads to the elimination of a water molecule and closes the ring to the 4*H*-3,1-benzothiazine skeleton (Scheme 1). This selective thioamide cyclization is promoted by the large shortage of electron density on the methylene carbon atom and the nucleophilic properties of sulfur.

STB as the starting reagent was obtained from 2,4-dihydroxybenzenecarbodithioic acid, which was prepared from resorcinol and CS_2 according to the modified Kolbe–Schmidt reaction. The process of 2,4-dihydroxybenzenecarbodithioic acid with $SOCl_2$ in diethyl ether gives STB.[19]



Scheme 1.



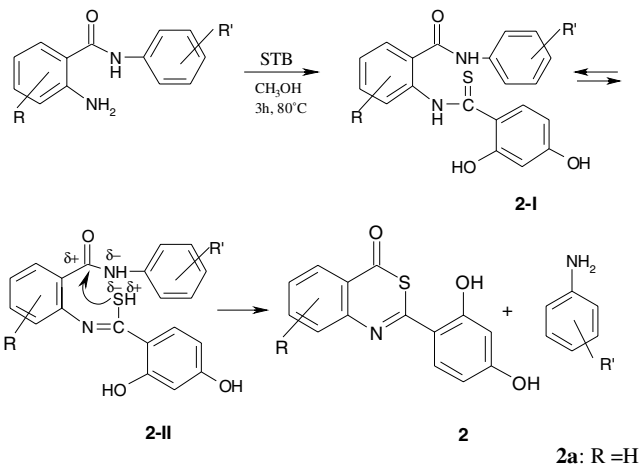
Scheme 2.

When 2-amino-*N*,*N*-disubstituted benzylamine was used in the reaction, the identical product (**1**) was obtained (Scheme 2). With this reagent an analogous mechanism, with the elimination of the corresponding amine, is proposed. As before, the reaction is promoted by low electron density on the carbon atom bearing the $-CH_2NR_1R_2$ group.

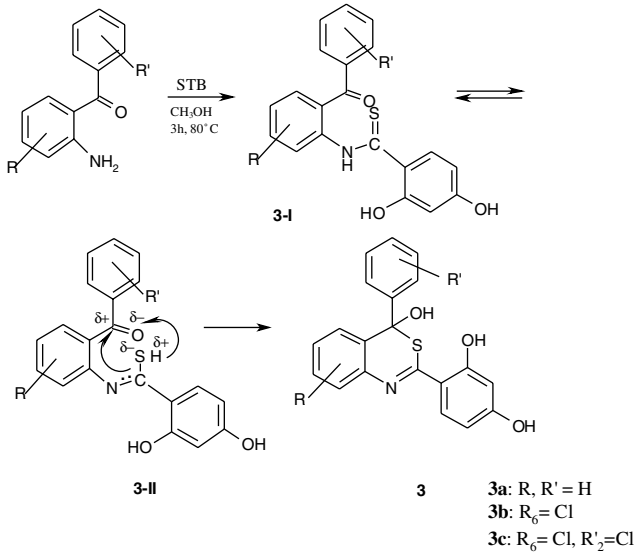
The reactions were performed in methanol by heating the reagents at the mixture boiling temperature for 2–3 h. Recrystallization from the methanol–water solution afforded a good yield of compounds with chromatographic purity. The structure of the compounds was established from elemental analyses, spectroscopic and mass spectra data. The ^{1}H NMR spectra of these compounds show, as expected, a singlet in the range δ 3.92–4.20 for the $-CH_2-$ moiety of the benzothiazine ring. There is a broad band in the range 9–11 ppm (2H) of –OH groups of resorcinol moiety.

STB reaction with 2-aminobenzanilides leads to 2-(2,4-dihydroxyphenyl)-4*H*-3,1-benzothiazin-4-ones (**2**) (Scheme 3). After formation of the local thioamide bond (**2-I**) and tautomerization (**2-II**), the attack of the –SH group on the neighbouring substituent takes place. The carbamide bond is cleaved, aniline released and the system cyclizes selectively to the fused heterocyclic ring (**2**).

STB reaction with substituted 2-aminobenzophenone leads to the preparation of 2,4-diaryl-4-hydroxy-4*H*-3,1-benzothiazine, racemic about the newly formed chiral centre on C*-4 of the heterocyclic ring (Scheme 4). As in the previous case, the system cyclizes to the benzothiazine ring after thioamide bond formation (**3-I**) and spontaneous tautomeric rearrangement to the equilibrium imido-thiol form (**3-II**). The intramolecular cyclization reaction proceeds according to the mechanism of hemithioketal formation. With the requirement of *ortho*-position of substituents ($-NH_2$ and –C(=O)-Ph), steric accessibility enables an intramolecular interaction of the $\rangle C{=}O$ and –SH groups (after tautomerization) (**3-II**), and formation of the 4*H*-3,1-benzthiazine ring



Scheme 3.

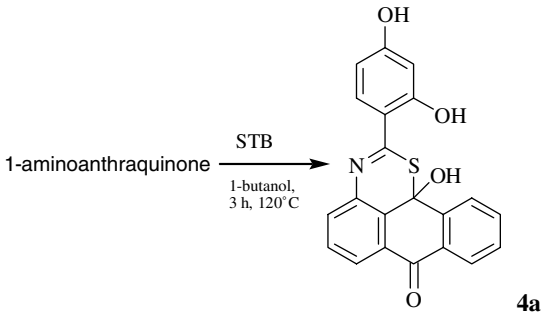


Scheme 4.

(**3**). The system is stabilized by an electron density scattering after the change of carbon atom hybridization from sp^2 (>C=O) to sp^3 of C-4 of the benzothiazine ring (Scheme 4). This reaction depends on amide proton activity and sulfur atom nucleophilicity, that is, on tautomeric equilibrium shift towards form **3-II**.

An X-ray diffraction analysis was performed to confirm the molecular structure of the products of the described process. The structure of compound **3b** is presented in Figure 1.[20] The conformation of the molecule is stabilized by an intramolecular hydrogen bond O(2)-H ··· N(1) (d (H–O) = 0.82 Å, d (H ··· N) = 1.77 Å, angle O(2)HN = 149°). Due to this interaction the 2,4-dihydroxyphenyl group is situated equatorially to the benzothiazine ring. The phenyl and hydroxyl substituents are situated pseudoaxially to that. The 4*H*-3, 1-benzothiazine ring assumes the conformation of a deformed semi-chair.

When 1-aminoanthraquinone was used as the reagent in the reaction, skeleton **4**, presented in Scheme 5, was obtained. In the IR spectrum, there is a strong bond in the range 1668 cm^{-1} characteristic of the =C=O group.



Scheme 5.

The anticancer activity of the synthesized compounds was evaluated in vitro. Human breast cancer T47D cells and cisplatin (comparatively) were used for studies. The cytotoxic activity in vitro was expressed as ID_{50} (μg/mL), the concentration of compound that inhibits proliferation rate of the tumour cells by 50% as compared to the control untreated cells. The results of the tests are presented in Table 1. All compounds studied show the antiproliferative effect ($ID_{50} \leqslant 36$ μg/mL), however, their activity is varied. ID_{50} value of the most active compound (**4a**) is lower than that of cisplatin, used as the reference system. At the same time compound **4a** meets the cytotoxic activity criterion ($ID_{50} \leqslant 4$ μg/mL) for new anticancer substances.[21] The antiproliferative effect of compounds **3** structure is promoted by the presence of chlorine atom (**3b**, **3c**), probably due to the strong electron-withdrawing properties, however influence on the degree of lipophilicity of derivatives cannot be excluded.

Using the dilution method, the minimal inhibitory concentration MIC (minimal inhibitory concentration caused full inhibition of growth) of individual compounds against four strains of moulds, five of yeasts and six of dermatophytes has been determined. These were either the reference strains of known sensitivity to antifungal drugs or the strains isolated directly from the clinical material. The results of in vitro screening of some compounds are summarized in Table 2. The MIC values against dermatophytes ranging from 0.49 to 7.8 μg/mL, against yeasts are ⩾3.9 μg/mL and

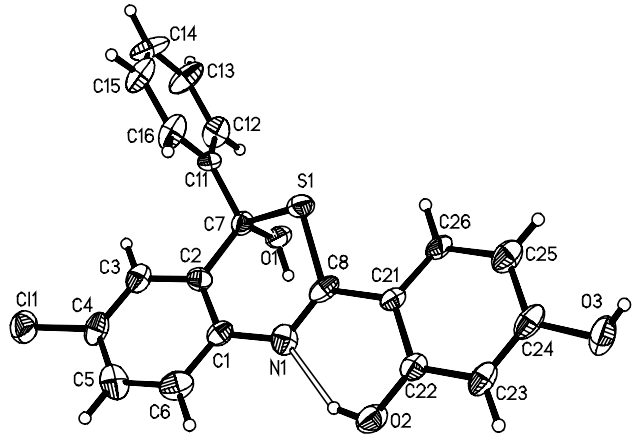


Figure 1. X-ray structure of 6-chloro-2-(2,4-dihydroxyphenyl)-4-hydroxy-4-phenyl-4*H*-3,1-benzothiazine (**3b**).

Table 1. Antiproliferative activity in vitro of compounds against the cells of a human breast cancer T47D line

Compound	ID_{50} (μg/mL)[a]	SD
1a	31.10	±9.15
2a	35.30	±1.10
3a	29.60	±15.38
3b	8.59	±1.22
3c	10.43	±5.61
4a	3.36	±1.39
Cisplatin	6.20	±1.50

[a] The cytotoxic activity was expressed as the concentration of compound that inhibits proliferation rate of the tumour cells by 50% as compared to the control untreated cells.

Table 2. Antifungal activity of compounds **1a**, **3a** and **3b** expressed by MIC[a] values and standards: nystatin and ketoconazole

No.	Strain	MIC (μg/mL)[a]				
		1a	**3b**	**3c**	Nystatin	Ketoconazole
1	*Penicillium* sp.	31.25	7.8	15.6	15.6	15.6
2	*Aspergillus fumigatus*	31.25	62.5	31.25	31.25	>31.25
3	*Aspergillus niger*	31.25	15.6	>31.25	31.25	>31.25
4	*Scopulariopsis brevicaulis*	15.6	15.6	31.25	31.25	>31.25
5	*Candida albicans*	15.6	3.9	15.6	1.98	>31.25
6	*Candida albicans ATCC 10231*	15.6	15.6	31.25	1.98	>31.25
7	*Cryptococcus neoformans*	15.6	15.6	15.6	3.9	3.9
8	*Geotrichum candidum*	15.6	31.25	15.6	31.25	3.9
9	*Trichosporon* sp.	15.6	15.6	15.6	31.25	7.8
10	*Epidermophyton floccosum*	3.9	0.49	3.9	0.98	0.06
11	*Microsporum gypseum*	3.9	1.98	7.8	1.98	3.9
12	*Trichophyton interdigitale*	1.98	0.99	7.8	0.98	0.12
13	*Trichophyton galline*	3.9	0.99	3.9	0.49	0.12
14	*Trichophyton mentagrophytes*	1.98	0.99	7.8	1.98	0.12
15	*Trichophyton rubrum*	3.9	1.98	3.9	0.98	0.24

[a] The minimal inhibitory concentration caused full inhibition of growth in relation to the control.

against moulds are $\geqslant$7.8 μg/mL. This indicates a significantly greater sensitivity of dermatophytes to the tested compounds compared with the other studied fungi. Similar tendencies were observed while studying the fungistatic activity of thiobenzanilides and thiadiazoles with a 2,4-dihydroxyphenyl moiety.[16,22] Assuming 15–25 μg/mL as the conventionally determined breakpoint for susceptibility to topical antifungal agents, compounds inhibit the growth of all strains of dermatophytes below these concentrations.[23] The most active compound **3b** meets this criterion also against *Penicillium* sp. and *Candida albicans*.

As biological tests are relative measures, nystatin and ketoconazole, tested under the same experimental conditions, were used as the reference system. MIC values of these antimycotic drugs were compared with those of the compounds (Table 2). It can be generally stated that the activity of compounds against strains of moulds and yeasts is at the level of the standards but significant differences in activity are observed against dermatophytes. The most active compound **3b** shows comparable activity in relation to that of nystatin and comparable to or weaker than that of ketoconazole.

Reported in the references results of biological studies of 4*H*-3,1-benzothiazines did not reveal fungistatic and bacteriostatic properties.[5] Therefore, the strength of antifungal effect of the presented group of compounds is probably connected with the presence of 2,4-dihydroxyphenyl moiety. This is also confirmed by a higher activity of variously substituted thioamides and thiobenzanilides with the 2,4-dihydroxyphenyl fragment compared with analogues presented in the literature. This substituent probably allows to reach a proper degree of hydrophobic–hydrophilic equlibrium of the molecule, promoting penetration through a fungus-protective barrier structure which is indicated by close relations between antifungal activity and various physicochemical parameters used for estimation of this character.[24] The introduction of balanced structures of appropriate lipophilicity leads to hydrophilic–hydrophobic equilibrium and facilitates the penetration of molecules through the cell membranes.

3. Conclusions

Summing up, relatively simple methods, involving the selective endocyclization of arylamides, were presented for the synthesis of various 4*H*-3,1-benzothiazine derivatives. The application of thioamides as intermediate products in reductive cyclization processes to obtain heterocyclic systems supports the proposed reaction mechanism.[25] Studies of antiproliferative activity of all synthesized compounds reveal an antitumour effect. Taking into account also the strong antifungal properties, particularly against dermatophytes, the research in this field will be continued. Therefore, the described method of synthesis will be applied to the preparation of new derivatives of 4*H*-3,1-benzothiazine with a 2,4-dihydroxyphenyl moiety.

4. Experimental

4.1. Chemistry

The melting point was determined with a Sanyo melting point apparatus. The elemental analysis was performed in order to determine the C, H and N contents (Perkin-Elmer 2400). Analyses (C, H and N) were within ±0.4% of the theoretical values. The oscillation spectra were recorded on a Perkin-Elmer FT-IR 1725X spectrophotometer (in potassium bromide). The spectra were recorded in the range of 600–4000 cm^{-1}. ^1H NMR spectra were recorded on a Bruker DRX 500 MHz instrument, standard TMS, solutions in deuterio DMSO, shift δ (ppm). The spectra MS (EI-70 eV) were recorded on a apparatus AMD-604.

4.2. 2-(2,4-Dihydroxyphenyl)-4*H*-3,1-benzothiazine (1a)

STB (0.0075 mol) and 2-aminobenzyl alcohol (Merck) (0.01 mol) were added to methanol (80 mL) and heated to boiling (2 h). The mixture was hot filtered. The filtrate was left at room temperature (24 h). The removed compound was recrystallized from aqueous (2:1) methanol (60 mL). Mp: 177–179 °C; ^{1}H NMR (DMSO-d_6, δ): 11.3–9.0 (2H, 2,4-COH, broad band), 4.17 (2H, CH_2, s); IR (KBr, cm^{-1}): 3392 and 3064 (OH), 1613 (C=N), 1558, 1506 (C=C), 1495, 1456, 1418, 1329, 1271, 1237, 1196 (C-OH), 1131, 1035, 1021, 982, 950, 895, 846, 800; MS (*m*/*z*, %): 257 (M^+, 100), 241 (8), 224 (40), 196 (19), 167 (6), 155 (4), 137 (4), 128 (10), 121 (11), 117 (10), 89 (4), 77 (8), 69 (3), 63 (2), 51 (4). Anal. Calcd for $C_{14}H_{11}NO_2S$ (257.31): C, 65.35; H, 4.31; N, 5.44. Found: C, 65.23; H, 4.33; N, 5.48.

4.3. 2-(2,4-Dihydroxyphenyl)-4*H*-3,1-benzothiazin-4-one (2a)

STB (0.0075 mol) and 2-aminobenzanilide (Alfa Aesar) (0.01 mol) were added to methanol (50 mL) and heated to boiling (3 h). The mixture was hot filtered and the filtrate was concentrated to dryness. The removed compound was washed by water and recrystallized from aqueous (3:1) methanol (60 mL). Mp: 245–247 °C; ^{1}H NMR (DMSO-d_6, δ): 11.71–11.65 (1H, 2-COH, d), 10.55 (1H, 4-COH, s); IR (KBr, cm^{-1}): 3284 (OH), 1660 (C=O), 1595 (C=N), 1510 (C=C), 1470, 1439, 1322, 1249, 1200 (C-OH), 1157, 1132, 1076, 981, 935, 893, 838, 802; MS (*m*/*z*, %): 271 (M^+, 100), 243 (4), 211 (51), 202 (4), 183 (13), 170 (2), 154 (9), 153 (1), 127 (2), 108 (18), 104 (3), 80 (3), 77 (7), 63 (3), 50 (4), 40 (6). Anal. Calcd for $C_{14}H_9NO_3S$ (271.29): C, 61.98; H, 3.34; N, 5.16. Found: C, 61.75; H, 3.36; N, 5.12.

4.4. 2-(2,4-Dihydroxyphenyl)-4-hydroxy-4-phenyl-4*H*-3,1-benzothiazine (3a)

STB (0.0075 mol) and 2-aminobenzophenone (Merck) (0.01 mol) were added to methanol (50 mL) and heated to boiling (3 h). The mixture was hot filtered and the filtrate was concentrated to dryness. The removed compound was washed by water and recrystallized from aqueous (3:1) methanol (80 mL). Mp: 110–111 °C; ^{1}H NMR (DMSO-d_6, δ): 14.00 (1H, C*-OH, s), 11.75–10.10 (2H, 2,4-COH, broad band); IR (KBr, cm^{-1}): 3249 (OH), 1618 (C=N), 1510 (C=C), 1478, 1447, 1326, 1248, 1195 (C-OH), 1128, 1075, 981, 942, 843, 803; MS (*m*/*z*, %): 333 (100), 300 (13), 283 (2), 272 (2), 260 (42), 258 (13), 256 (45), 230 (3), 224 (15), 196 (20), 192 (28), 180 (4), 169 (19), 160 (44), 136 (5), 128 (25), 105 (4), 96 (5), 77 (9), 64 (41). Anal. Calcd for $C_{20}H_{15}NO_3S$ (349.40): C, 68.75; H, 4.33; N, 4.01. Found: C, 68.53; H, 4.35; N, 4.04.

4.5. 6-Chloro-2-(2,4-dihydroxyphenyl)-4-hydroxy-4-phenyl-4*H*-3,1-benzothiazine (3b)

STB (0.0075 mol) and 2-amino-5-chlorobenzophenone (Merck) (0.01 mol) were added to methanol (80 mL). Pyridine (2 mL) was added to the mixture and heated to boiling (3 h). The mixture was hot filtered and the filtrate was left at room temperature (24 h). The removed compound was recrystallized from aqueous (3:1) methanol (40 mL). Mp: 200–201 °C; ^{1}H NMR (DMSO-d_6, δ): 14.26 (1H, C*-OH, s), 11.75–11.50 (1H, 2-COH, d), 10.40 (1H, 4-COH, s); IR (KBr, cm^{-1}): 3404 (OH), 1620 (C=N), 1511 (C=C), 1469, 1392, 1320, 1286, 1247, 1194 (C-OH), 1172, 1132, 1097 (C-Cl), 1023, 983, 955, 916, 887, 848, 823; MS (*m*/*z*, %): 383 (M^+, 1), 350 (2), 308 (4), 280 (40), 278 (100), 246 (3), 230 (3), 208 (7), 152 (6), 137 (2), 105 (7), 77 (9). Anal. Calcd for $C_{20}H_{14}ClNO_3S$ (383.85): C, 62.58; H, 3.68; N, 3.65. Found: C, 62.78; H, 3.70; N, 3.63.

4.6. 6-Chloro-4-(2-chlorophenyl)-4-hydroxy-2-(2,4-dihydroxyphenyl)-4*H*-3,1-benzothiazine (3c)

STB (0.0075 mol) and 2-amino-2′,5-dichlorobenzophenone (Merck) (0.01 mol) were added to methanol (50 mL) and heated to boiling (3 h). The mixture was hot filtered and water (100 mL) was added to the filtrate. The removed compound was washed by water and recrystallized from aqueous (5:1) methanol (60 mL). Mp: 220–221 °C; H NMR (DMSO-d_6, δ): 13.99 (1H, C*-OH, s), 10.45 (1H, 4-COH, s); IR (KBr, cm^{-1}): 3399 (OH), 1619 (C=N), 1512 (C=C), 1469, 1308, 1273, 1194 (C-OH), 1130, 1087 (C-Cl), 1050, 975, 944, 882, 847; MS (*m*/*z*, %): 418 (M^+, 5), 416 (8), 399 (90), 401 (86), 380 (10), 360 (12), 344 (8), 330 (4), 296 (23), 278 (6), 261 (16), 246 (4), 230 (5), 220 (3), 195 (6), 186 (5), 177 (4), 164 (4), 139 (47), 111 (11), 75 (5), 69 (4). Anal. Calcd for $C_{20}H_{13}Cl_2NO_3S$ (418.29): C, 57.43; H, 3.13; N, 3.35. Found: C, 57.16; H, 3.11; N, 3.37.

4.7. 11b-Hydroxy-2-(2,4-dihydroxyphenyl)anthra[1,9-de][1,3]thiazin-7(11b*H*)-one (4a)

STB (0.0075 mol) and 1-aminoanthraquinone (Merck) (0.01 mol) were added to *n*-butanol (50 mL) and heated to boiling (3 h). The mixture was hot filtered and the filtrate was left at room temperature (24 h). The filtrate was concentrated to dryness and recrystallized from methanol (50 mL). Mp: 228–230 °C; ^{1}H NMR (DMSO-d_6, δ): 13.00 (1H, C*-OH, s), 11.96–11.66 (1H, 2-COH, d), 10.78–10.27 (1H, 4-COH, m); IR (KBr, cm^{-1}): 3422 and 3309 (OH), 1668 (C=O), 1636 (C=N), 1605, 1591 (C=C), 1543, 1480, 1454, 1414, 1383, 1338, 1283, 1166 (C-OH), 998, 979, 934, 890, 879, 832, 802 cm^{-1}; MS (*m*/*z*, %): 375 (M^+, 9), 373 (100), 359 (14), 345 (2), 316 (39), 299 (3), 290 (5), 284 (8), 260 (5), 247 (2), 223 (80), 195 (7), 139 (6), 137 (19), 105 (4), 81 (6), 77 (8), 63 (5), 44 (8), 39 (96). Anal. Calcd for $C_{21}H_{13}NO_4S$ (375.40): C, 67.19; H, 3.49; N, 3.73. Found: C, 67.39; H, 3.51; N, 3.71.

4.8. Antiproliferative assay in vitro

The solutions of compounds (1 mg/mL) were prepared ex tempore by dissolving the substance in 100 μL 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 human breast cancer cell line T47D obtained from the American Type Culture Collection (Rockville, MD, USA) stored in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wrocław, Poland, was applied for the test. Twenty-four hours before the addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, USA) at a density of 10^4 cells/well. Cell line was maintained in the opti-MEM medium supplemented with glutamine (2 mM) (Gibko, Warsaw, Poland), streptomycin (50 μg/mL), penicillin (50 U/mL) (Polfa, Tarchomin, Poland) and 5% foetal calf serum (Gibko, Grand Island, USA). The cells were incubated at 37 °C in a humid atmosphere saturated with 5% CO_2. The SRB test measuring the cell proliferation inhibition in in vitro culture was applied.[26] The cytotoxicity assay was performed after 72 h exposure of the cultured cells at a concentration ranging from 0.1 to 100 μg/mL of the tested agents. The cells attached to the plastic were fixed with cold 50% TCA (trichloroacetic acid, Aldrich-Chemie, Germany) added on 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 microtitre plate reader Uniskan II (Labsystems, Helsinki, Finland). The compounds were tested in triplicate per experiment. The experiments were repeated at least 3 times.

4.9. Antifungal assay in vitro

Microorganisms were multiplied on the slants developed from the Muller–Hinton agar containing 4% glucose (pH 5.6) and from the analogous Muller–Hinton broth. The tested compounds were dissolved in methanol. Different amounts of solutions were added to the accurately measured, dissolved and cooled to 45 °C agar medium, and then mixed and emptied onto Petri plates. The medium of more and more decreasing concentrations ranging from 31.25 to 0.015 μg/mL was obtained. The medium containing 0.5 mL of the substance also had 5% of methanol. After solidification, the plates were dried, and after the 0.02 mL culture (10^4 colony-forming units (cfu) of fungi) had been sprayed, the plates were incubated for 2–10 days at 22 °C. At the same time the sensitivity of the strains to methanol was determined. The activity of ketoconazole and nystatin against all fungi was also estimated. The presented results were obtained from three independent measurements. The investigations were carried out in the Department of Pharmaceutical Microbiology, Medical University, Lublin.

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