

Synthesis, spectroscopic characterization, and anticancer activity of new 10-substituted 1,6-diazaphenothiazines

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Received: 6 July 2015 / Accepted: 4 July 2016
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Abstract New phenothiazine derivatives as 10-substituted dipyridothiazines of the 1,6-diazaphenothiazine structure were obtained in the cyclization reaction of 3-amino-3'-nitro-2,2'-dipyridinyl sulfide and 3,3'-dinitro-2,2'-dipyridinyl disulfide, and in the reaction of 2-chloro-3-ntropyridine with sodium 3-amino-2-pyridinethiolate followed by various alkylation and arylation reactions. The reaction of the thiazine ring formation ran via the Smiles rearrangement of the S-N type. As the alkylation reactions could proceed at the thiazine, azine or both nitrogen atoms, the product structure elucidation was based on the 2D NMR (Rotating-frame Overhauser Effect Spectroscopy, Correlated Spectroscopy, Heteronuclear Single Quantum Coherence, and Heteronuclear Multiple Bond Correlation) spectra of the *N*-methylated product. Some 10-substituted 1,6-diazaphenothiazines (**5**, **10**, **12**, **13**) were at least anticancer active against melanoma C-32 and breast cancer MCF-7 cell lines as a reference drug – cisplatin. The monoazaphenothiazine drug, prothipendyl, turned out to be less active than least 6 derivatives of the 1,6-diazaphenothiazine structure.

Keywords Phenothiazines · Dipyridothiazines · NMR structure elucidation · 2D NMR spectra · The Smiles rearrangement · Anticancer activity

Introduction

Tricyclic phenothiazines (dibenzo-1,4-thiazines) are important class of heterocycles possessing significant biological activities and interesting chemical features. Classical 10-substituted phenothiazines with the aminoalkyl groups at the nitrogen atom have been for many years valuable drugs exhibiting neuroleptic, antihistaminic, antitussive, and antiemetic activities (Gupta and Kumar, 1988). They are relatively easy-obtainable, inexpensive, and low toxic, and they can be valuable source for searching new drugs of other biological activities. The chemical structure modifications of these compounds were carried out mainly by introduction of new substituents at the thiazine nitrogen atom and substitution of one or two benzene rings with homoaromatic and heteroaromatic rings. Such modifications are expected to change not only potency but also types of activities. Both classical and modified phenothiazines are found to exhibit very promising anticancer, antibacterial, antifungal, anti-inflammatory, and multidrug resistance reversal activities, summarized recently in the review articles and chapters in monographs (Motohashi et al., 2000, 2006; Mitchell, 2006; Dasgupta et al., 2008; Aaron et al., 2009; Sudeshna and Parimal, 2010; Pluta et al., 2011; Wesołowska, 2011; Jaszczyszyn et al., 2012). They show also a potential benefit in treatment of Alzheimer's, Creutzfeldt-Jakob's, and AIDS-associated diseases (Mosnaim et al., 2006; González-Muñoz et al., 2010).

The last type of the modification with azine rings lead to formation of azaphenothiazines. Our strategy for modification

Electronic supplementary material The online version of this article (doi:10.1007/s00044-016-1646-3) contains supplementary material, which is available to authorized users.

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of the phenothiazine structure is based on the introduction of two pyridine rings instead of the benzene ones to form various dipyrido[1,4]thiazines. We found new dipyrido[1,4]thiazines of the 1,8- and 2,7-diazaphenothiazine structures to exhibit promising anticancer activity against lung cancers HOP-62 and HOP-92, colon cancers COLO 205, HCT-116 and SW-948, renal cancers RXF393 and A498, and leukemia HL-60 (TB) and L-1210 (Pluta et al., 2010; Morak-Młodawska et al., 2015). 10*H*-2,7-diazaphenothiazine shows also immunosuppressant, inhibiting both humoral and cellular immune responses, and antioxidant properties (Zimecki et al., 2009; Morak-Młodawska et al., 2010).

It is well known that the synthesis of phenothiazine and azaphenothiazine ring system may proceed via 1,4-thiazine ring formation with the use of diphenyl sulfides, phenyl azinyl sulfides or diazinyl sulfides directly in the Ullmann cyclization or indirectly through the Smiles rearrangement of the S-N type to diphenylamines, phenylazinylamines, and diazinylamines followed by cyclization. During the rearrangement, the phenyl or azinyl part migrates from the sulfur atom to the nitrogen atom (Pluta et al., 2009; Silberg et al., 2006). There only two reports of double Smiles rearrangement during those syntheses (Morak et al., 2002; Morak-Młodawska et al., 2012).

The synthesis of 10-substituted derivatives from 10*H*-diazaphenothiazines by the alkylation of the thiazine nitrogen atom can be disturbed by alkylation of the azine nitrogen atom.

For those reasons, the unquestioned elucidation of the structure of the direct product, NH-azaphenothiazine, and its *N*-substituted derivatives is crucial.

In continuation of those studies we have worked out an efficient synthesis of another type of dipyridothiazines, 10*H*-1,6-diazaphenothiazine, and the transformation of this parent compound into 10-substituted derivatives, possessing alkyl, arylalkyl, heteroaryl and dialkylaminoalkyl, and

imidoalkyl groups. In this work, we discuss the synthesis and the structure elucidation of the NH- and *N*-alkyl-1,6-diazaphenothiazines, and their anticancer activity.

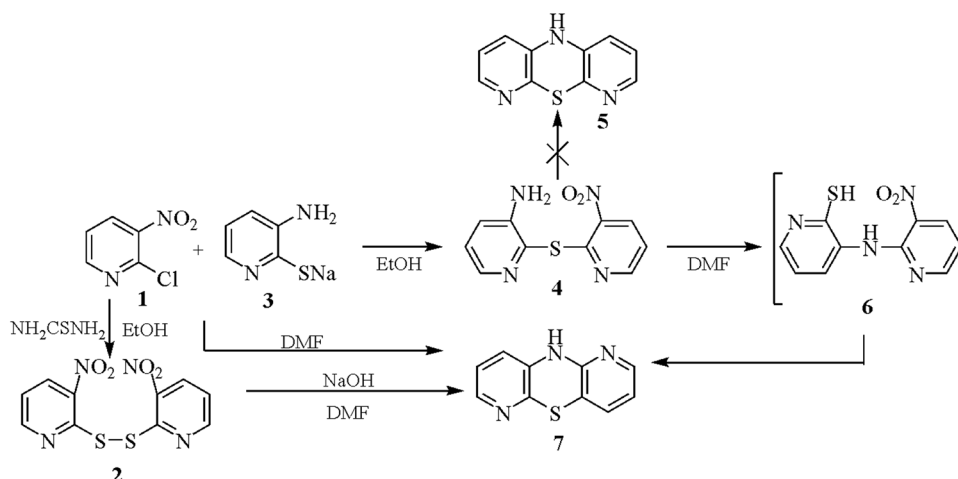
Results and discussion

Chemistry

The possibility of the Smiles rearrangement depends on the sulfide structure and reaction conditions. The most often the rearrangement proceeds under basic conditions (sodium hydroxide in ethanol), rarely under neutral or acidic media. It is sometimes difficult to state if the rearrangement took place as the rearranged and non-rearranged products can have the same or similar structure. In the last case, the structure difference is in the location of a substituent or a nitrogen atom (in the azaphenothiazine structure) (Pluta et al., 2009).

In our case, 3-amino-3'-nitro-2,2'-dipyridinyl sulfide **4** (obtained from 2-chloro-3-nitropyridine **1** and sodium 3-amino-2-pyridinethiolate **3** in ethanol) heated in refluxing *N,N*-dimethylformamide (DMF) solution did not undergo cyclization to symmetrical 10*H*-4,6-diazaphenothiazine **5** (giving only three aromatic proton signals in the ¹H NMR spectrum) but to 10*H*-1,6-diazaphenothiazine **7** in 90 % yield. It means that the synthesis proceeded through the Smiles rearrangement to dipyridinylamine **6** (which was not isolated, Scheme 1). We found 3,3'-dinitro-2,2'-dipyridinyl disulfide **2** (obtained from compound **1** to give compound **3**) to be quite a good substrate to form 1,6-diazaphenothiazine **7** (in 72 % yield) in boiling DMF in the presence of sodium hydroxide. This is very useful synthesis because disulfide can be obtained from commercially available 2-chloro-3-nitropyridine **1** in 89 % yield. The same phenothiazine product was obtained directly (in 64 % yield) from pyridines **1** and **3** in boiling DMF. It is not the first synthesis of 10*H*-1,6-

Scheme 1 Synthesis of 10*H*-1,6-diazaphenothiazine **7**



diazaphenothiazine **7** as Rodig and coworkers obtained compound **7** in low yield (43 %) in cyclization of 3-acetyl-amino-3'-nitro-2,2'-dipyridinyl sulfide in ethanolic solution of potassium hydroxide and in very good yield (92 %) in cyclization of dipyridinylamine **6** in DMSO-ethanol solution of potassium hydroxide. In both cases the reactions substrates were obtained in two steps using 2-chloro-3-nitropyridine **1** (Rodig et al., 1966).

The next step was transformation of compound **7** into *N*-substituted derivatives mainly by alkylation. Although the alkylation of phenothiazines proceeds mainly at the thiazine nitrogen atom, there are a few reports on the alkylation of azaphenothiazines at the azine nitrogen atom giving azaphenothiazinium salts and neutral *N*-alkylazaphenothiazines (Clarke et al., 1961; Werle et al., 1962; Pappalardo et al., 1973; Carter and Cheeseman, 1977; Saari et al., 1983). We studied the reaction of compound **7** with methyl iodide in dry DMF in the presence of sodium hydride. The methylated product possessed only one methyl group (observed in the ^1H NMR spectrum) without the ammonium function what could point at the structure **8**. To exclude alternative neutral *N*-methylazaphenothiazine structure i.e. 1-methyl-1,6-diazaphenothiazine **8A**, we recorded 2D NMR (Rotating-frame Overhauser Effect Spectroscopy (ROESY), Correlated Spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC)) spectra of the *N*-methyl product. The ROESY experiment with irradiation of the methyl protons at 3.40 ppm showed the proximity of the methyl group to the proton at 6.98 ppm

(γ -pyridinyl proton) and pointed at the structure **8**. Only spatial ^1H - ^1H connectivity with a proton at about 8 ppm (α -pyridinyl proton) could point at the structure **8A**. The full proton signal assignment was achieved by study of other proton spatial proximity (ROESY) and ^1H - ^1H connectivities (COSY). The signal at 6.98 ppm was assigned as H-9 proton. The confirmation of the proton assignment came from the ^{13}C NMR spectrum which was solved by the use of HSQC and HMBC spectra indicating the ^{13}C - ^1H relationship. The HSQC spectrum showed which proton was bonded to the carbon atom (the C-H relationship through one bond, $^1J_{\text{C,H}}$ connectivity) and the HMBC spectrum indicated the C-H relationship through three (predominantly), two and four (exceptionally) bonds ($^3J_{\text{C,H}}$, $^2J_{\text{C,H}}$ and $^4J_{\text{C,H}}$ connectivities). Selected spatial proton-proton proximity, proton-proton and proton-carbon connectivities for compound **8** were shown in Scheme 2. The all ^1H - ^1H and ^1H - ^{13}C connectivities were included in Table 1. The resulted product was identified as 10-methyldipyrido[2,3-b;2',3'-e][1,4]thiazine **8**. The ^1H and ^{13}C NMR spectra of the rest compounds were solved using the HSQC and HMBC experiments.

The parent product **7** was transformed into new derivatives possessing the allyl (**9**), propargyl (**10**), benzyl (**11**), nitropyridinyl (**12**), phthalimidopropyl (**17**) and the dialkylaminoethyl (**13**–**16**, with cyclic and non-cyclic amine group) substituents in the reactions of appropriate dialkylaminoethyl halides in the presence of base (NaH, NaOH, *t*-BuOK) in neutral solvents. The propargyl derivative **10** was converted into the dialkylaminobutynyl derivatives **18**

Scheme 2 The 2D NMR experiments for compound **8**: ROESY, COSY, HSQC, and HMBC (selected connectivities)

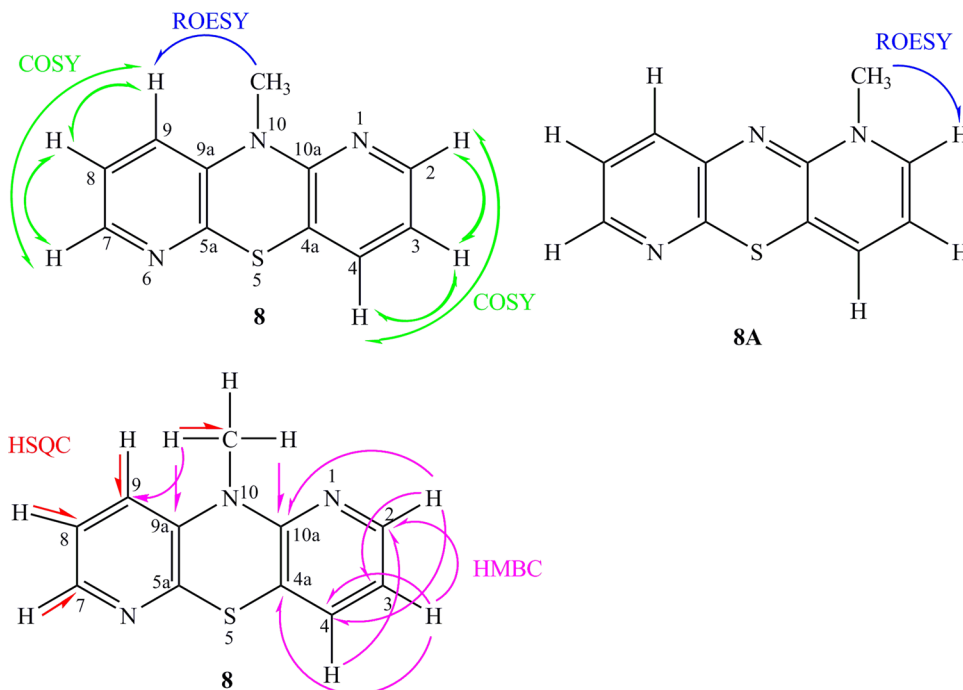
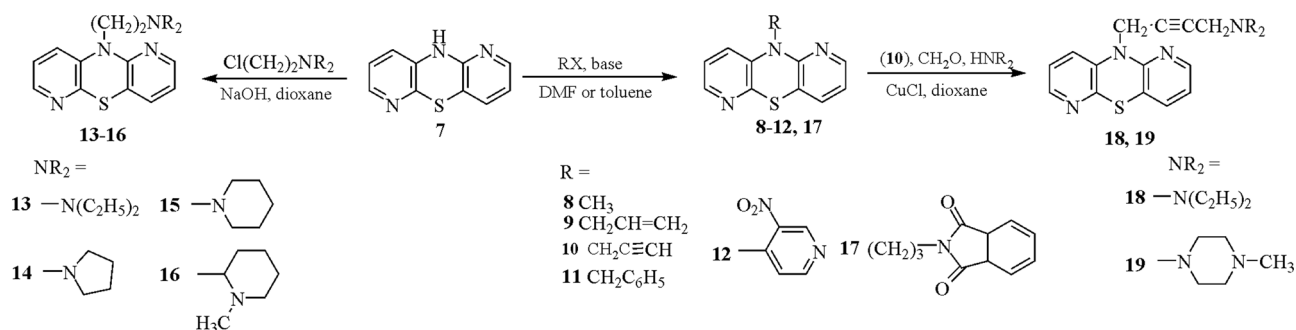


Table 1 The ^1H and ^{13}C NMR assignment, and full proton–proton and proton–carbon connectivities (ROESY, COZY, HSQC, and HMBC) for 10-methyl-1,6-diazaphenothiazine **8**

^1H NMR δ (ppm)	ROESY	COSY	^{13}C NMR δ (ppm)	HSQC	HMBC
CH_3 3.40	3.40–6.98	—	CH_3 32.84	3.40–32.84	3.40–120.31/139.73/153.49
H_3 6.81	6.98–3.40/7.05	6.98–7.05	C_{4a} 116.62	6.81–118.19	6.81–116.62/134.66/145.29
H_9 6.98	6.81–7.31	6.81–7.31/8.04	C_3 118.19	6.98–122.11	6.98–142.62/144.76
H_8 7.05	7.05–6.98	7.05–7.31/6.98	C_9 120.31	7.05–122.11	7.05–139.73/142.62
H_4 7.31	7.31–6.81	7.31–6.81	C_8 122.11	7.31–134.66	7.31–145.29/153.49
H_7 8.02	—	8.02–7.05	C_4 134.66	8.02–142.62	8.02–122.11/144.76/120.31
H_2 8.04	—	8.04–6.81	C_{9a} 139.73	8.04–145.29	8.04–118.19/134.66/153.49
			C_7 142.62		
			C_{5a} 144.76		
			C_2 145.29		
			C_{10a} 153.49		

**Scheme 3** Synthesis of 10-substituted 1,6-diazaphenothiazines

and **19** (with the triple bond) via the Mannich reaction with formaldehyde and secondary amine (non-cyclic and cyclic) in the presence of copper(I) chloride in dioxane (Scheme 3).

Anticancer activity

The anticancer activity of 1,6-diazaphenothiazines **7–19** was investigated in vitro using cultured glioblastoma SNB-19, melanoma C-32 and breast cancer MCF-7 cell lines. Normal human fibroblast (HFF-1) cell line was used as a control and cisplatin as a reference drug. To compare the influence of the nitrogen atoms in the azaphenothiazine system on the anticancer activity, the classical monoazaphenothiazine drug, prothipendyl (10-dimethylaminopropyl-1-azaphenothiazine), was also tested. The tested compounds exhibited different activities against the cell lines. The MCF-7 cell line was found as very sensitive for most compounds. Eight derivatives exhibited good anticancer activity with $\text{IC}_{50} < 10 \mu\text{g/mL}$ (Table 2). The most active ($\text{IC}_{50} < 5 \mu\text{g/mL}$) were compounds **7**, **10** and **12** with the hydrogen atom, and the propargyl and nitropyridinyl groups. Those

compounds were more active than cisplatin. Compound **19** (with the methylpiperazinylbutynyl group) was as active as cisplatin and compounds **13**, and **17** (with the diethylaminoethyl and phthalimidopropyl groups) were slightly less active.

The parent compound **7** and derivative **13** (with the diethylaminoethyl group) were as active as cisplatin against melanoma C-32 cell line. The SNB-19 cell line was the most resistant for the tested compounds. The most active derivative **9** (with the allyl group) exhibited IC_{50} close to $20 \mu\text{g/mL}$.

Compounds **11**, **15** and **18** (with the benzyl, piperidylethyl and diethylaminobutynyl groups) were completely inactive against all cell lines with $\text{IC}_{50} > 50 \mu\text{g/mL}$.

Prothipendyl, containing only one pyridine ring, was moderately active only against the MCF-7 cell line but about 5–6 times less active than the most active diazaphenothiazines. It worth noting that the most active compounds **7**, **12** and **13** (together with six other compounds) were non-toxic against normal fibroblasts (HFF-1) with the $\text{IC}_{50} > 50 \mu\text{g/mL}$ whereas cisplatin turned out to be toxic.

Table 2 The anticancer activity of derivatives of 1,6-diazaphenothiazines

No.	Anticancer activity IC ₅₀ (μg/mL)			
	SNB-19	C-32	MCF-7	HFF-1
7	32.8	7.5	4.8	>50
8	33.3	>50	9.1	>50
9	18.9	44.1	42.3	31.6
10	>50	27.1	3.9	6.1
11	>50	>50	>50	>50
12	28.2	32.0	4.6	>50
13	24.2	6.6	8.2	>50
14	>50	>50	10.7	>50
15	>50	>50	>50	>50
16	31.3	16.3	9.4	10.0
17	>50	>50	8.2	>50
18	>50	>50	>50	>50
19	49.1	35.3	7.5	46.6
Prothipendyl	48.7	>50	23.2	>50
Cisplatin	7.7	7.8	7.4	8.2

Conclusion

We report here synthesis of new 10-substituted 1,6-diazaphenothiazines. Parent compound, 10*H*-1,6-diazaphenothiazine **7**, was obtained in three ways from appropriate dipyridinyl sulfide and disulfide, and a pair of 2,3-disubstituted pyridines. The thiazine ring formation ran via the Smiles rearrangement of the S-N type. The parent compound was transformed into 10-substituted derivatives with the alkyl, heteroaryl, dialkylaminoalkyl, dialkylaminoalkynyl and imidoalkyl groups in the alkylation and heteroarylation reactions. As the alkylation reactions could proceed at the thiazine, azine or both nitrogen atoms, the product structure elucidation was based on the 2D NMR (ROESY, COSY, HSQC, and HMBC) spectra of the *N*-methylated product. Some 1,6-diazaphenothiazines (**7**, **10**, **12**, **13**) were at least anticancer active against melanoma C-32 and breast cancer MCF-7 cell lines as a reference drug – cisplatin. Monoazaphenothiazine drug, prothipendyl, turned out to be less active than at least six derivatives of 1,6-diazaphenothiazines against all three cancer cell lines.

Experimental

Chemistry

Melting points were determined in open capillary tubes on a Boetius melting point apparatus and are uncorrected. The ¹H, ¹³C NMR, COSY, NOESY, HSQC, HMBC spectra

were recorded on a Bruker Ascend™ 600 spectrometer at 600 MHz in deuteriochloroform with tetramethylsilane as the internal standard. The ¹³C NMR spectra were recorded at 150 MHz. Electron impact mass spectra (EI MS) and fast atom bombardment mass spectra (FAB MS, in glycerol) were run on a Finnigan MAT 95 spectrometer at 70 eV. The thin layer chromatography was performed on silica gel 60 F₂₅₄ (Merck 1.05735) with CHCl₃–EtOH (5:1 and 10:1 v/v) and on aluminum oxide 60 F₂₅₄ neutral (type E) (Merck 1.05581) with CHCl₃–EtOH (10:1 v/v) as eluents.

Synthesis of 3,3'-dinitro-2,2'-dipyridinyl disulfide (**2**)

A solution of 2-chloro-3-nitropyridine (**1**) (158 mg, 1 mmol) and thiourea (152 mg, 2 mmol) in ethanol (10 mL) was refluxed for 3 h. After cooling the resulting crystals were filtered off, washed with water and air dried to give 3,3'-dinitro-2,2'-dipyridinyl disulfide (**2**) as orange needles (140 mg, 89 %) m.p. 249–250 °C. ¹H NMR (CDCl₃) δ: 7.32 (dd, *J* = 7.8 Hz, *J* = 4.2 Hz, 2H, H₅, H_{5'}), 8.57 (dd, *J* = 7.8 Hz, *J* = 1.2 Hz, 2H, H₄, H_{4'}), 8.61 (dd, *J* = 4.2 Hz, *J* = 1.2 Hz, 2H, H₆, H_{6'}). ¹³C NMR (CDCl₃) δ: 120.82 (2CH, C₅, C_{5'}), 133.80 (2CH, C₄, C_{4'}), 146.71 (2CH, C₃, C_{3'}), 153.54 (2CH, C₆, C_{6'}), 161.02 (2C, C₂, C_{2'}, CS). EI MS *m/z*: 310 (M, 5), 156 (M+1-NO₂C₅H₃N, 25) 92 (100). Anal. calcd. for: C₁₀H₆N₄O₄S₂, C 38.71; H 1.95, N 18.06. Found: C 38.51, H 1.63, N 17.99.

Synthesis of sodium 3-amino-2-pyridinethiolate (**3**)

To a solution of 3,3'-dinitro-2,2'-dipyridinyl disulfide (**2**) (310 mg, 1 mmol) in absolute ethanol (30 mL) two tablets of NaBH₄ (10 mmol) were added carefully and the solution was refluxed for 2 h. After cooling the solvent was removed and evaporated in vacuo. The dry residue was recrystallized from ethanol, yielding brown crystals of sodium 3-amino-2-pyridinethiolate (**3**) (230 mg, 71 %), m.p. > 260 °C. After acidification with 10 % solution of HCl, 3-aminopyridine-2 (1*H*)-thione was obtained, m.p. 131–132 °C (lit. (Rodig et al., 1964) 131–132 °C).

Synthesis of 3-amino-3'-nitro-2,2'-dipyridinyl sulfide (**4**)

To a solution of sodium 3-amino-2-pyridinethiolate (**3**) (148 mg, 1 mmol) in dry ethanol (10 mL) 2-chloro-3-nitropyridine (**1**) (158 mg, 1 mmol) was added. The mixture was stirred at room temperature for 3 h and next the resulting brown crystals were filtered off, washed with ethanol, air dried and recrystallized from ethanol, yielding 3-amino-3'-nitro-2,2'-dipyridinyl sulfide (**4**) as yellow needles (220 mg, 89 %), m.p. 166 °C (lit. [27] 167–168 °C)).

¹H NMR (CDCl₃) δ: 7.12 (m, 1H), 7.24 (m, 1H), 7.49 (m, 1H), 8.13 (broad s, 2H, NH₂), 8.23 (m, 1H), 8.53 (m, 1H),

8.64 (m, 1H). ^1H NMR (CDCl_3) δ : 4.27 (broad s, 2H, NH_2), 7.12 (m, 1H, H_4), 7.24 (m, 2H, H_5 , H_5'), 8.14 (m, 1H, H_4'), 8.55 (m, 2H, H_6 , H_6'). ^{13}C NMR (CDCl_3) δ : 119.87 (CH, C_5), 122.56 (CH, C_4), 125.80 (CH, C_5'), 133.80 (CH, C_6), 135.66 (C, C_3), 140.79 (CH, C_4'), 142.29 (C, C_2), 146.81 (C, C_3'), 153.62 (CH, C_6'), 156.52 (C, C_2'). EI MS m/z : 248 (M, 25), 202 (M+1- NO_2 100). Anal. calcd. for: $\text{C}_{10}\text{H}_8\text{N}_4\text{O}_2\text{S}$, C 48.38, H 3.25, N 22.57. Found: C 48.42, H 3.39, N 22.51.

Synthesis of 10H-1,6-diazaphenothiazine (7)

From 2-chloro-3-nitropyridine (**1**) and sodium 3-amino-2-pyridinethiolate (**3**) To a solution of sodium 3-amino-4-pyridinethiolate (**3**) (148 mg, 1 mmol) in dry DMF (10 mL) 2-chloro-3-nitropyridine (**1**) (158 mg, 1 mmol) was added. The mixture was stirred at room temperature for 1 h and next was refluxed for 4 h. After cooling the reaction mixture was evaporated in vacuo. The dry residue was dissolved in CHCl_3 and purified by column chromatography (aluminum oxide, CHCl_3) to give 10H-1,6-diazaphenothiazine (**7**) as beige needles (EtOH) (130 mg, 64 %), m.p. 191–192 °C (lit. (Rodig et al., 1966) 223–225 °C). ^1H NMR (CDCl_3) δ : 6.68 (dd, J = 7.8 Hz, J = 1.2 Hz, 1H, H_9), 6.72 (1H, NH), 6.73 (dd, J = 7.2 Hz, J = 4.8 Hz, 1H, H_3), 6.88 (dd, J = 7.2 Hz, J = 4.8 Hz, 1H, H_8), 7.18 (dd, 1H, J = 7.8 Hz, J = 1.2 Hz, H_4), 7.81 (dd, J = 4.8 Hz, J = 1.2 Hz, 1H, H_7), 7.92 (dd, J = 4.8 Hz, J = 1.2 Hz, 1H, H_2). ^{13}C NMR (CDCl_3) δ : 114.49 (C_{4a}), 118.54 (C_3), 120.21 (C_9), 122.20 (C_8), 134.31 (C_4), 136.31 (C_{9a}), 141.37 (C_{5a}), 143.28 (C_7), 145.45 (C_2), 151.22 (C_{10a}). EI MS m/z : 201 (M, 100). Anal. calcd. for: $\text{C}_{10}\text{H}_7\text{N}_3\text{S}$, C 59.68, H 3.51, N 20.88. Found: C 59.51, H 3.53, N 20.82.

In cyclization of 3-amino-3'-nitro-2,2'-dipyridinyl sulfide (**4**) The brown solution of 3-amino-3'-nitro-2,2'-dipyridinyl sulfide (**4**) (124 mg, 0.5 mmol) in dry DMF (5 mL) was refluxed for 4 h. After cooling the reaction mixture was evaporated in vacuo. The dry residue was dissolved in CHCl_3 and purified by column chromatography (aluminum oxide, CHCl_3) to give 10H-1,6-diazaphenothiazine (**7**) (90 mg, 90 %).

In cyclization of 3,3'-dinitro-2,2'-dipyridinyl disulfide (**2**) To a solution of 3,3'-dinitro-4,4'-dipyridinyl disulfide (**2**) (310 mg, 1 mmol) in dry DMF (10 mL) NaOH (120 mg, 3 mmol) was added and refluxed for 48 h. After cooling the reaction mixture was evaporated in vacuo. The dry residue was dissolved in CHCl_3 and purified by column chromatography (aluminum oxide, CHCl_3) to give 10H-1,6-diazaphenothiazine (**7**) (144 mg, 72 %).

Synthesis of 10-substituted 1,6-diazaphenothiazines 8–12

To a solution of 10H-1,6-diazaphenothiazine (**7**) (100 mg, 0.5 mmol) in dry DMF (5 mL) NaH (24 mg, 1 mmol, 60 %

NaH in mineral oil was washed out with hexane) was added. The reaction mixture was stirred at room temperature for 1 h and then alkyl or heteroaryl halides (methyl iodide, allyl bromide, benzyl chloride, 4-chloro-3-pyridine, 1.5 mmol) was added and the stirring was continued for 24 h. The mixture was poured into water (15 mL), extracted with CHCl_3 (3 \times 10 mL) and dried using anhydrous Na_2SO_4 . The obtained product was purified by column chromatography (aluminum oxide, CHCl_3) to give:

10-Methyl-1,6-diazaphenothiazine (**8**) (98 mg, 91 %); light brown needles (EtOH), m.p. 105–106 °C ^1H NMR (CDCl_3) δ : 3.40 (s, 3H, CH_3), 6.81 (dd, J = 7.2 Hz, J = 4.8 Hz, 1H, H_3), 6.98 (d, J = 7.8 Hz, 1H, H_9), 7.05 (dd, J = 7.8 Hz, J = 4.8 Hz, 1H, H_8), 7.31 (dd, 1H, J = 7.2 Hz, J = 1.2 Hz, H_4), 8.02 (dd, J = 4.2 Hz, J = 1.2 Hz, 1H, H_7), 8.04 (dd, J = 4.2 Hz, J = 1.2 Hz, 1H, H_2). ^{13}C NMR (CDCl_3) δ : 32.84 (NCH_3), 116.62 (C_{4a}), 118.19 (C_3), 120.31 (C_9), 122.11 (C_8), 134.66 (C_4), 139.73 (C_{9a}), 142.62 (C_7), 144.76 (C_{5a}), 145.29 (C_2), 153.49 (C_{10a}). EI MS m/z : 215 (M, 100), 200 (M- CH_3 , 45). Anal. calcd. for: $\text{C}_{11}\text{H}_9\text{N}_3\text{S}$ C 61.37, H 4.21, N 19.52. Found: C 61.24, H 4.21, N 19.49.

10-Allyl-1,6-diazaphenothiazine (**9**) (103 mg, 86 %); a dark yellow oil ^1H NMR (CDCl_3) δ : 4.63 (m, 2H, NCH_2), 5.27 (m, 2H, $=\text{CH}_2$), 5.99 (m, 1H, CH), 6.76 (dd, J = 7.5 Hz, J = 4.8 Hz, 1H, H_3), 6.97 (m, 2H, H_8 , H_9), 7.23 (dd, J = 7.5 Hz, J = 1.5 Hz, 1H, H_4), 7.96 (m, 2H, H_2 , H_7). ^{13}C NMR (CDCl_3) δ : 47.82 (CH, CH_2), 117.20 (CH, $\text{CH}_2=$), 132.58 (CH, $\text{CH}=$), 115.77 (C, C_{4a}), 118.48 (CH, C_3), 121.77 (CH, C_9), 122.09 (CH, C_8), 134.62 (CH, C_4), 139.02 (C, C_{9a}), 141.40 (CH, C_7), 143.98 (C, C_{5a}), 145.29 (CH, C_2), 152.04 (C, C_{10a}). EI MS m/z : 241 (M, 55), 200 (M- CH_2CHCH_2 , 100). Anal. calcd. for: $\text{C}_{13}\text{H}_{11}\text{N}_3\text{S}$ C 64.70, H 4.59, N 17.41. Found: 64.67, H 4.56, N 17.32.

10-Benzyl-1,6-diazaphenothiazine (**11**) (95 mg, 65 %); a dark yellow oil ^1H NMR (CDCl_3) δ : 5.29 (s, 2H, CH_2), 6.68 (m, 1H, H_3), 6.88 (m, 2H, H_9 , H_8), 7.27 (dd, J = 7.2 Hz, J = 1.4 Hz, 1H, H_4), 7.38 (m, 5H, C_6H_5), 7.92 (m, 2H, H_2 , H_7). ^{13}C NMR (CDCl_3) δ : 48.78 (CH, CH_2), 116.22 (C, C_{4a}), 118.43 (CH, C_3), 121.45 (CH, C_9), 121.83 (CH, C_8), 126.45 (2CH, o -CH), 126.98 (CH, p -CH), 128.82 (2CH, m -CH), 134.46 (CH, C_4), 136.60 (C, CCH_2), 138.44 (C, C_{9a}), 142.66 (CH, C_7), 144.38 (C, C_{5a}), 145.34 (CH, C_2), 152.52 (C, C_{10a}). EI MS m/z : 291 (M, 30), 200 (M- $\text{CH}_2\text{C}_6\text{H}_5$, 100). Anal. calcd. for: $\text{C}_{17}\text{H}_{13}\text{N}_3\text{S}$ C 70.08, H 4.50, N 14.42. Found: C 70.04, H 4.54, N 14.30.

10-(3'-Nitro-4'-pyridinyl)-1,6-diazaphenothiazine (**12**) (125 mg, 75 %); as red needles (EtOH), m.p. 167–169 °C ^1H NMR (CDCl_3) δ : 6.32 (d, 1H), 6.81 (m, 1H), 6.89 (m, 1H), 7.33 (d, 1H, H_4), 7.53 (d, 1H), 7.72 (d, 1H), 8.06 (d, 1H), 9.04 (d, 1H), 9.42 (s, 1H). ^{13}C NMR (CDCl_3) δ : 116.07 (C, C_{4a}), 119.74 (CH, C_3), 121.95 (CH, C_8), 123.21

(CH, C₉), 126.36 (CH, C_{5'}), 135.08 (CH, C₄), 138.19 (C, C_{9a}), 141.06 (C, C_{4'}), 144.01 (C, C_{5a}), 144.06 (CH, C₇), 144.68 (CH, C₂), 146.09 (C, C_{3'}), 146.85 (C, C_{2'}), 149.50 (C, C_{10a}), 156.24 (CH, C_{6'}), 145.34 (CH, C₂), 152.52 (C, C_{10a}). EI MS *m/z*: 323 (M, 60), 277 (M+1-NO₂, 100), 200 (M-NO₂C₆H₄, 10). Anal. calcd. for: C₁₅H₉N₅O₂S C 55.72, H 2.81, N 21.66 Found: C 56.04, H 2.96, N 22.01.

Synthesis of 10-propargyl-1,6-diazaphenothiazines (**10**)

To a suspension of 10*H*-1,6-diazaphenothiazine (**7**) (100 mg, 0.5 mmol) in DMF (10 mL) potassium *tert*-butoxide (80 mg, 0.72 mmol) was added. The mixture was stirred at room temperature for 1 h. Then to the solution 80 % solution of propargyl bromide (80 mg, 0.64 mmol) in dry toluene (0.12 mL) was added dropwise. The solution stirred at room temperature for 24 h and poured into water (20 mL), extracted with methylene chloride (20 mL), dried with anhydrous Na₂SO₄, evaporated to the brown oil. The residue was purified by column chromatography (silica gel, CHCl₃) to yield 10-propargyl-1,6-diazaphenothiazine (**10**) (83 mg, 70 %); dark yellow needles (EtOH), m.p. 96–97 °C.

¹H NMR: δ 2.31 (t, *J* = 2.4 Hz, 1H), 4.69 (d, *J* = 2.4 Hz, 2H), 6.84 (dd, *J* = 7.5 Hz, *J* = 5.1 Hz, 1H, H₃), 7.06 (dd, *J* = 7.5 Hz, *J* = 5.1 Hz, 1H, H₈), 7.31 (m, 2H, H₄, H₉), 8.10 (d, *J* = 5.5 Hz, 1H, H₇), 8.3 (dd, *J* = 5.1 Hz, *J* = 1.2 Hz, 1H, H₇), 8.15 (dd, *J* = 5.1 Hz, *J* = 1.3 Hz, 1H, H₂). ¹³C NMR (CDCl₃) δ: 35.03 (CH, NCH₂), 72.50 (CH, CH₂CCH), 79.10 (C, CH₂CCH), 116.40 (C, C_{4a}), 118.69 (CH, C₃), 121.04 (CH, C₉), 122.02 (CH, C₈), 134.65 (CH, C₄), 137.84 (C, C_{9a}), 143.13 (CH, C₇), 144.54 (C, C_{5a}), 145.34 (CH, C₂), 151.87 (C, C_{10a}). EI MS: 239 (M, 90), 200 (M-CH₂CCH, 100). Anal. calcd. for: C₁₃H₉N₃S C 65.25, H 3.79, N 17.56. Found: C 65.21, H 3.74, N 17.38.

Synthesis of 10-substituted 1,6-diazaphenothiazines **13–16**

To a solution of 10*H*-1,6-diazaphenothiazine (**7**) (100 mg, 0.5 mmol) in dry dioxane (10 mL) NaOH (200 mg, 5 mmol) was added. The mixture was refluxed for 1.5 h and hydrochloride of dialkylaminoalkyl chloride (2-diethylaminoethyl) and hydrochloride of cycloaminoethyl chloride 1-(2-chloroethyl)pyrrolidine, 1-(2-chloroethyl)piperidine, 2-(2-chloroethyl)-1-methylpiperidine 1.5 mmol) was added. The reaction mixture was refluxed for 24 h. After cooling dioxane was evaporated in vacuo and residue was dissolved in CHCl₃ (10 mL). The extracts were washed with water, dried with anhydrous Na₂SO₄ and evaporated in vacuo. The obtained product was purified by column chromatography (aluminum oxide, CH₂Cl₂) to give:

10-(2'-Diethylaminoethyl)-1,6-diazaphenothiazine (**13**) (110 mg, 72 %); a beige oil ¹H NMR: δ 1.06 (t, *J* = 7.2 Hz,

6H, 2CH₃), 2.65 (q, *J* = 7.2 Hz, 4H, 2CH₂), 2.78 (t, *J* = 7.2 Hz, 2H, CH₂), 4.08 (t, *J* = 7.2 Hz, 2H, CH₂), 6.72 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₃), 6.97 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₈), 7.13 (dd, *J* = 7.2 Hz, *J* = 1.2 Hz, 1H, H₄), 7.19 (dd, *J* = 7.2 Hz, *J* = 1.2 Hz, 1H, H₉), 7.94 (m, 2H, H₂, H₇). ¹³C NMR (CDCl₃) δ: 11.96 (2CH, 2CH₃), 44.00 (CH, NCH₂), 47.69 (2C, 2CH₂CH₃), 48.98 (CH, NCH₂), 116.30 (C, C_{4a}), 118.07 (CH, C₃), 120.66 (CH, C₉), 122.00 (CH, C₈), 134.25 (CH, C₄), 138.77 (C, C_{9a}), 142.39 (CH, C₇), 144.39 (C, C_{5a}), 145.13 (CH, C₂), 152.42 (C, C_{10a}). FAB MS *m/z*: 301 (M+1, 20), 228 (M+1-NC₄H₁₀, 100), 200 (M+1-C₂H₄NC₄H₁₀, 25). Anal. calcd. for: C₁₆H₂₀N₄S C 63.97; H 6.71; N 18.65. Found: C 63.88; H 6.74; N 18.43.

10-(2'-Pyrrolidinylethyl)-1,6-diazaphenothiazine (**14**) (110 mg, 75 %); a beige oil ¹H NMR (CDCl₃) δ: 1.48 (m, 2H, CH₂), 1.60 (m, 4H, 2CH₂), 2.52 (m, 4H, 2CH₂), 2.68 (t, *J* = 7.5 Hz, 2H, CH₂), 4.15 (t, *J* = 7.5 Hz, 2H, NCH₂), 6.73 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₃), 6.96 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₈), 7.12 (m, 2H, H₄, H₉), 7.94 (m, 2H, H₂, H₇). ¹³C NMR (CDCl₃) δ: 23.35 (2CH, 2CH₂), 40.90 (CH, NCH₂), 49.42 (CH, NCH₂), 53.57 (2CH, 2CH₂), 115.99 (C, C_{4a}), 118.66 (CH, C₃), 120.11 (CH, C₉), 122.01 (CH, C₈), 135.78 (CH, C₄), 137.94 (C, C_{9a}), 143.15 (CH, C₇), 144.66 (C, C_{5a}), 145.36 (CH, C₂), 152.29 (C, C_{10a}). FAB MS *m/z*: 299 (M+1, 100), 202 (M+1-C₂H₄NC₄H₈, 29). Anal. calcd. for: C₁₆H₁₈N₄S C 64.40; H 6.08; N 18.78. Found: C 64.25; H 6.05; N 18.55.

10-(2'-Piperidinyloethyl)-1,6-diazaphenothiazine (**15**) (112 mg, 72 %); a beige oil ¹H NMR (CDCl₃) δ: 1.48 (m, 2H, CH₂), 1.61 (m, 4H, 2CH₂), 2.52 (m, 4H, 2CH₂), 2.68 (t, *J* = 6.8 Hz, 2H, CH₂), 4.13 (t, *J* = 6.8 Hz, 2H, NCH₂), 6.73 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₃), 6.96 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₈), 7.12 (m, 2H, H₄, H₉), 7.94 (m, 2H, H₂, H₇). ¹³C NMR (CDCl₃) δ: 23.86 (CH, CH₂), 25.32 (2CH, 2CH₂), 42.47 (CH, NCH₂), 54.41 (2CH, 2CH₂), 54.86 (CH, NCH₂), 116.43 (C, C_{4a}), 118.21 (CH, C₃), 120.91 (CH, C₉), 122.22 (CH, C₈), 134.34 (CH, C₄), 138.50 (C, C_{9a}), 142.59 (CH, C₇), 144.22 (C, C_{5a}), 145.10 (CH, C₂), 152.26 (C, C_{10a}). FAB MS *m/z*: 313 (M+1, 100), 202 (M+1-C₂H₄NC₅H₁₀, 20). Anal. calcd. for: C₁₇H₂₀N₄S C 65.35; H 6.45; N 17.93. Found: C 65.22; H 6.47; N 17.80.

10-(1'-Methyl-2'-piperidinyloethyl)-1,6-diazaphenothiazine (**16**) (119 mg, 74 %); a beige oil ¹H NMR (CDCl₃) δ: 1.30–2.15 (m, 7H), 2.38 (s, 3H, NCH₃), 2.94 (m, 1H, CH), 4.02 (m, 2H, NCH₂), 6.73 (dd, *J* = 7.6 Hz, *J* = 5.1 Hz, 1H, H₃), 6.96 (m, 2H, H₈, H₄), 7.20 (m, 1H, H₉), 7.94 (m, 2H, H₂, H₇). ¹³C NMR (CDCl₃) δ: 23.93 (CH, CH₂), 25.11 (CH, CH₂), 28.58 (CH, CH₂), 30.38 (CH, CH₂), 41.00 (CH, NCH₃), 42.50 (CH, CH₂), 56.79 (CH, CH), 62.34 (CH, NCH₂), 116.38 (C, C_{4a}), 118.10 (CH, C₃), 120.28 (CH, C₉), 122.02 (CH, C₈), 134.37 (CH, C₄), 138.57 (C, C_{9a}), 142.41 (CH, C₇), 144.61 (C, C_{5a}), 145.14 (CH, C₂), 152.47 (C,

C_{10a}). FAB MS. 327 (M+H, 80), 313 (M+1-CH₃, 100). Anal. calcd. for: C₁₈H₂₂N₄S C 66.22; H 6.79; N 17.16. Found: C 66.17; H 6.75; N 17.05.

Synthesis of 10-phthalimidopropyl-1,6-diazaphenothiazines (17)

To a stirred solution of 10H-1,6-diazaphenothiazine (7) (100 mg, 0.5 mmol) in dry toluene (20 mL) NaH (0.12 g, 5 mmol, washed out with hexane) was added. The mixture was stirred for 30 min at room temperature, then refluxed for 1 h and a solution of *N*-(3-bromopropyl)phthalimide (405 mg, 1.5 mmol) in toluene (10 mL) was added. The mixture was refluxed for 48 h. After cooling the resulted solid was filtered off, toluene was evaporated in vacuo and the residue was purified by column chromatography (aluminum oxide, CHCl₃) to give 10-(3'-phthalimidopropyl)-1,6-diazaphenothiazine (17) (115 mg, 73 %), reddish needles (Et₂O), m.p. 42–44 °C. ¹H NMR (CDCl₃) δ: 2.28 (m, 2H, CH₂), 3.43 (t, *J* = 6.1 Hz, 2H, NCH₂), 3.87 (t, *J* = 6.0 Hz, 2H, NCH₂), 6.72 (m, 2H, H₃, H₉), 6.88 (m, 1H, H₈), 7.18 (m, 1H, H₄), 7.74 (m, 2H_{phthalimide}), 7.79 (m, 1H, H₇), 7.87 (m, 2H_{phthalimide}) 7.92 (m, 2H, H₂). ¹³C NMR (CDCl₃) δ: 29.82 (CH, CH₂), 31.65 (CH, NCH₂), 36.74 (CH, NCH₂), 114.95 (C, C_{4a}), 118.40 (CH, C₉), 120.42 (CH, C₃), 122.26 (CH, C₈), 123.36 (2CH, 2CH_{phthalimide}), 132.00 (2C, 2C_{phthalimide}), 134.09 (2CH, 2CH_{phthalimide}), 135.66 (CH, C₄), 136.11 (C, C_{10a}), 141.23 (C, C_{9a}), 143.39 (CH, C₂), 144.68 (CH, C₇), 151.16 (C, C_{5a}), 168.29 (2C, 2CO). FAB MS *m/z*: 389 (M+1, 100), 201 (M+1-(CH₂)₃N(CO)₂C₆H₄, 30). Anal. calcd. for C₂₁H₁₆N₄O₂S: C 64.93, H 4.15, N 14.42. Found: C 64.84, H 4.19, N 14.31.

General procedure for synthesis of 10-dialkylaminobutynyl-1,6-diazaphenothiazines (18, 19)

A mixture of 10-propargyl-1,6-diazaphenothiazine (10) (100 mg, 0.5 mmol), paraformaldehyde (0.5 mmol), amine (0.7 mmol) and copper(I) chloride (catalytic amount) in peroxide-free, dry dioxane (10 mL) was heated with continuous stirring at 70–80 °C for 3 h. After cooling water (20 mL) was add and mixture was extracted with chloroform, dried with anhydrous Na₂SO₄, and evaporated in vacuo. The dry residue was dissolved in CH₂Cl₂ and purified by column chromatography (aluminum oxide, CH₂Cl₂) to give:

10-(4-diethylaminobut-2-ynyl)-1,6-diazaphenothiazine (18) (130 mg, 80 %); a yellowish oil ¹H NMR (CDCl₃) δ: 1.03 (t, *J* = 7.2 Hz, 6H, 2CH₃), 2.50 (q, *J* = 7.2 Hz, 4H, 2 NCH₂), 3.42 (s, 2H, CH₂), 4.70 (s, 2H, CH₂), 6.79 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₃), 7.03 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₈), 7.33 (m, 2H, H₄, H₉), 8.03 (m, 2H, H₂, H₇). ¹³C NMR (CDCl₃) δ: 11.68 (2CH, 2CH₃), 35.18 (CH, CH₂), 40.65

(2CH, 2CH₂), 47.45 (CH, NCH₂), 79.01 (C, CH₂C≡), 80.01 (C, CH₂C≡), 116.47 (C, C_{4a}), 118.68 (CH, C₃), 121.07 (CH, C₉), 121.90 (CH, C₈), 134.68 (CH, C₄), 137.78 (C, C_{9a}), 143.13 (CH, C₇), 144.66 (C, C_{5a}), 145.16 (CH, C₂), 151.92 (C, C_{10a}). FAB MS *m/z*: 325 (M+1, 15), 252 (M+1-C₄H₁₀N, 100), 201 (M+1-C₈H₁₄N, 20). Anal. calcd. for: C₁₈H₂₀N₄S C 66.63, H 6.21, N 17.27. Found: C 66.41, H 6.27, N 17.12.

10-[4-(4-Methylpiperazin-1-yl)but-2-ynyl]-1,6-diazaphenothiazine (19) (120 mg, 69 %); a yellowish oil ¹H NMR (CDCl₃) δ: 1.25 (s, 3H, NCH₃), 2.69 (m, 8H, 4CH₂), 3.32 (s, 2H, CH₂), 4.57 (s, 2H, CH₂), 6.80 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₃), 7.09 (dd, *J* = 7.8 Hz, *J* = 4.8 Hz, 1H, H₈), 7.33 (m, 2H, H₄, H₉), 8.03 (m, 2H, H₂, H₇). ¹³C NMR (CDCl₃) δ: 23.83 (2CH, 2CH₂), 25.75 (2CH, 2CH₂), 35.17 (CH, CH₂), 47.98 (CH, NCH₃), 53.27 (CH, CH₂), 79.80 (C, CH₂C≡), 80.10 (C, CH₂C≡), 116.26 (C, C_{4a}), 118.51 (CH, C₃), 121.33 (CH, C₉), 121.86 (CH, C₈), 134.63 (CH, C₄), 137.90 (C, C_{9a}), 142.95 (CH, C₇), 144.50 (C, C_{5a}), 145.22 (C₂), 151.98 (C_{10a}). FAB MS *m/z*: 351 (M+1, 100), 201 (M+1-C₉H₁₅N₂, 40). Anal. calcd. for: C₁₉H₂₁N₅S C 64.93, H 6.02, N 19.93. Found: C 64.77, H 5.94 N 19.79.

Cytotoxic and antiproliferative effects in vitro

Cell culture

Compounds were evaluated for their anticancer activity using three cultured cell lines: SNB-19 (human glioblastoma, DSMZ - German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), C-32 (human amelanotic melanoma, ATCC - American Type Culture Collection, Manassas, VA, USA), MCF-7 (human breast cancer, ATCC, Manassas, VA, USA) and HFF-1 (human fibroblast cell line, ATCC, Manassas, VA, USA). The cultured cells were kept at 37 °C and 5 % CO₂. The cells were seeded (1 × 10⁴ cells/well/100 μL Dulbecco's modified Eagle's medium supplemented with 10 % FCS and streptomycin and penicillin) using 96-well plates (Corning).

Cell proliferation and viability

In recent years tetrazolium salts have been described to be used for the measurement of cell proliferation and viability. The tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture. The formazan dye produced by metabolically active cells is quantified by a scanning enzyme-linked immunosorbent assay reader by measuring

the absorbance of the dye solution at appropriate wavelengths ($\lambda = 420\text{--}480\text{ nm}$ with a reference wavelength $\lambda = 600\text{ nm}$).

WST-1 assay

The WST-1 assay (Roche Diagnostics, Mannheim, Germany) was used to evaluate the effect of compounds on the number of cells in cultures, which as the cytotoxic effect of the tested compounds and their influence on the proliferation of cells. After exposure to tested compounds (at concentrations between 0 and 100 $\mu\text{g/mL}$) for 72 h, cells were incubated with WST-1 (10 μL) for 1 h, and the absorbance of the samples against a background control was read at 450 nm with a reference wavelength $\lambda = 600\text{ nm}$ using a microplate reader UVM340 (Biogenet). Results are expressed as means of at least two independent experiments performed in triplicate.

Acknowledgments The work was supported by the Medical University of Silesia (grant KNW-1-004/K/4/0).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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