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2-(Substituted phenyl)amino analogs of 1-methoxyspirobrassinol methyl ether: Synthesis and anticancer activity

Peter Kutschy [a,*], Aneta Salayová [a], Zuzana Čurillová [a], Tibor Kožár [b], Roman Mezencev [c], Ján Mojžiš [d], Martina Pilátová [d], Eva Balentová [a], Pavel Pazdera [e], Marián Sabol [f], Michaela Zburová [a]

[a] Department of Organic Chemistry, Institute of Chemical Sciences, Faculty of Science, P.J. Šafárik University, Moyzesova 11, 040 01 Košice, Slovak Republic
[b] Department of Biophysics, Institute of Experimental Physics, Slovak Academy of Sciences, Watsonova 47, 040 01 Košice, Slovak Republic
[c] Georgia Institute of Technology, College of Science, School of Biology, 315 Ferst Drive, Atlanta, GA 30332, USA
[d] Department of Pharmacology, Faculty of Medicine, P.J. Šafárik University, SNP 1, 040 66 Košice, Slovak Republic
[e] Centre for Syntheses at Sustainable Conditions and Their Management, Department of Chemistry, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic
[f] Department of Microbiology, Faculty of Medicine, P.J. Šafárik University, SNP 1, 040 66 Košice, Slovak Republic

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ABSTRACT

New analogs of indole phytoalexin 1-methoxyspirobrassinol methyl ether have been designed by replacement of its 2-methoxy group with 2-(substituted phenyl)amino group. Synthesized by spirocyclization methodology, *trans*- and *cis*-diastereoisomers of target compounds were isolated and evaluated as potential anticancer and antimicrobial agents. Their molecular geometries were refined by ab initio minimizations. Pharmacophore modeling and QSAR studies were performed in order to correlate their molecular structure and biological activity.

1. Introduction

Indole-derived sulfur-containing phytoalexins are structurally unique natural products isolated from economically and dietary important plants of the family *Cruciferae* (syn. *Brassicaceae*). Cruciferous indole phytoalexins have been reviewed with regard to their isolation, occurrence, synthesis, biosynthesis, biotransformation, and their biological activity and role in plant defenses.[1–5] Besides their antimicrobial properties, several indole phytoalexins also exhibit anticancer activity.[4] Specifically, brassinin (**1**, Fig. 1), cyclobrassinin (**3**), and (±)-spirobrassinin [(±)-**5**] demonstrated cancer chemopreventive activity in the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced precancerous lesions in mouse mammary gland organ culture.[6] In addition, brassinin (**1**) exhibited dose-dependent inhibition of DMBA-induced and TPA-promoted skin tumorigenesis in CD-1 mice.[7] Cytotoxic effects against various solid tumor and leukemia cell lines in vitro were reported for brassinin (**1**),[8] (±)-spirobrassinin [(±)-**5**],[8,9] camalexin (**4**),[10] 1-methoxyspirobrassinol (**6**, mixture of diastereoisomers that easily isomerize owing to their hemiaminal structure[11]),[9] 1-methoxybrassinin (**2**),[9] and (±)-1-methoxyspirobrassinin [(±)-**7**)].[9] While the anticancer properties of natural (*S*)-(−)-spirobrassinin (**5**) have not yet been studied, the natural isomers of (*R*)-(+)-1-methoxyspirobrassinin (**7**) and (2*R*,3*R*)-(−)-1-methoxyspirobrassinol methyl ether [(−)-**8**] were recently obtained in their individual enantiomeric forms and (2*R*,3*R*)-(−)-1-methoxyspirobrassinol methyl ether [(−)-**8**] was found to be a more potent inhibitor of Jurkat cell proliferation than its (2*S*,3*S*)-(+)-enantiomer [(+)-**8**] and racemic (±)-**8**. On the other hand, in the case of 1-methoxyspirobrassinin (**7**) there was no difference in potencies between the enantiomers, and both displayed overall weak activity against Jurkat cells.[12] Lately, the *trans*- and *cis*-diastereoisomers[13] of 2-piperidyl analogs were evaluated on the NCI_{60} panel of human cancer cell lines. Introduction of a piperidyl moiety resulted in enhanced antiproliferative activity with the most potent anticancer effect exhibited by *trans*-(±)-**9** against leukemic cell line CCRF-CEM with $IC_{50} = 0.0263\ \mu mol \times L^{-1}$.[14]

Even though the spiroindoline structures are present in many biologically attractive natural products,[15] the anticancer and antimicrobial properties of 1-methoxyspiroindoline phytoalexins and their analogs are still almost unexplored. For this reason, we chose 1-methoxyspirobrassinol methyl ether (**8**), as a lead compound and designed its 2-(substituted phenyl)amino analogs via the replacement of 2-methoxy by 2-(substituted phenyl)amino group. The introduction of 2-(substituted phenyl)amino substituent enables

* Corresponding author. Tel.: +421 552341256; fax: +421 55 62 22124.
E-mail address: peter.kutschy@upjs.sk (P. Kutschy).

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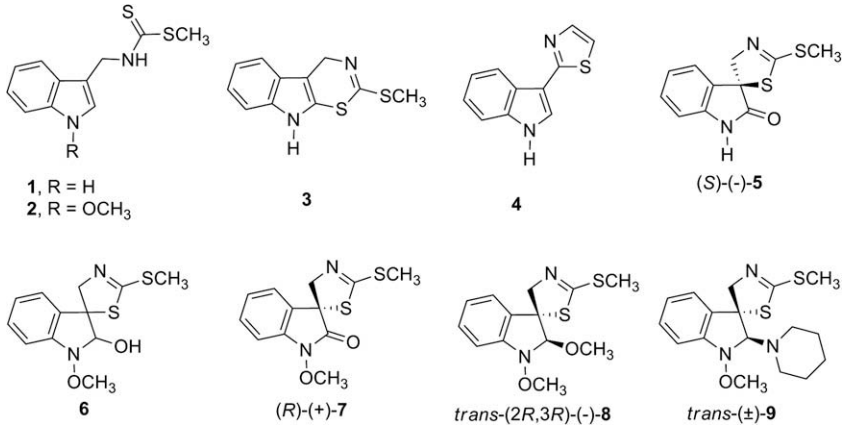


Figure 1. Structures of selected indole phytoalexins.

the tuning of physico-chemical properties and hence the biological properties as well. Furthermore, both secondary amino and aromatic moieties appear very frequently in drugs and other biologically active compounds. In addition, compared to the natural lead **8** with no hydrogen bond donor, the secondary amino group introduces a H-bond donor into designed analogs that might be beneficial for drug-target interactions. We determined cytostatic/cytotoxic and antimicrobial activities of the synthesized compounds, identified molecular fragments responsible for anticancer activities on different cell lines, and predicted topoisomerase II as their prospective drug target.

2. Results and discussion

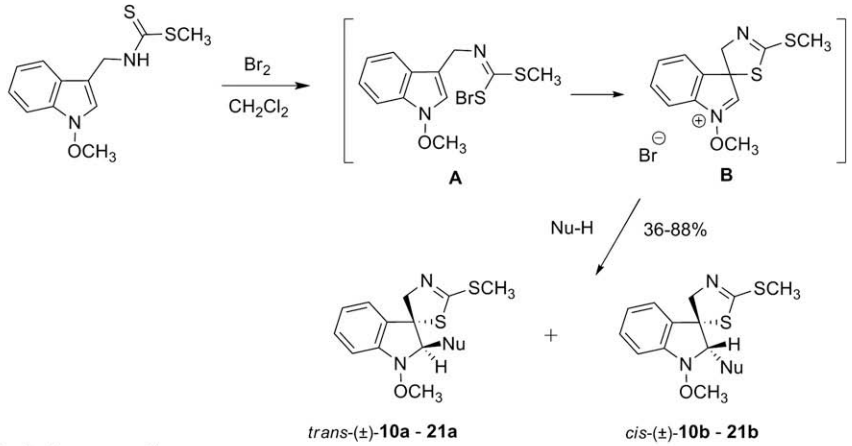
2.1. Chemistry

The dioxane dibromide mediated spirocyclization of 1-methoxybrassinin (**2**) in dioxane developed in our laboratory[16] opened a new way for synthesis of 1-methoxyspiroindoline phytoalexins and various related derivatives. Starting 1-methoxybrassinin (**2**) can be prepared in small quantities from unstable 1-methoxyindole-3-ylmethylamine (obtained by the reduction of 1-methoxyindole-3-carboxaldehyde oxime) by the reaction with CS_2 and CH_3I in a mixture of pyridine and triethylamine as a solvent in 60–64% yield.[17,18] If we performed the reaction with a quantity of starting oxime over 120 mg, the yields were not higher than 40%. Replacement of the basic solvent by methanol improved the overall yield to about 70%, shortened the reaction time from 24 h to 15 min and is suitable for a higher quantity of starting oxime. With a sufficient amount of 1-methoxybrassinin (**2**) available, syntheses of new analogs of 1-methoxyspirobrassinol methyl ether can be studied more conveniently since the pure 1-methoxybrasinin (**2**) is a stable oily compound that can be stored for several months without decomposition. Spirocylization of 1-methoxybrassinin (**2**) with bromine in dry dichloromethane, which appeared to be more suitable compared to dioxane,[13,14] proceeds via sulfenyl bromide **A** which after electrophilic cyclization produces 1-methoxyspiroindoleninium intermediate **B**. After trapping by nucleophiles it afforded products **10a**–**21b** (Scheme 1). In this way, the target spiroindolines were obtained by efficient electrophilic-nucleophilic difunctionalization of the indole moiety. First we studied the synthesis of 2-methylamino derivative, isosteric to natural product **8**. In this case only the *cis*-isomer **10b** was isolated as a pure substance since the *trans*-diastereoisomer **10a** owing to its isomerization during the separation on alumina or silica gel was obtained only as a mixture with *cis*-diastereoisomer in the ratio 4:1 in favor of *trans*-isomer. Next we prepared 2-phenoxy (**11a** and **11b**) and isosteric 2-phenylamino (**12a** and **12b**) derivatives that afforded isolable diastereoisomers. Since the activity of 2-phenylamino derivative was notably higher, in the next study substituted phenylamino groups were introduced into position 2 by reaction of methoxyiminium intermediate **B** with substituted anilines as nucleophiles. Selection of substituents was inspired by the Topliss operational scheme[19] enabling the choice of the substituents with expected higher and lower activity compared to the parent unsubstituted lead **12** based on Hammett electronic, Taft steric and Hantzsch lipophilic substituent parameters. Thus a suitable series of compounds for a SAR study was obtained. In all cases both diastereoisomers were isolated (Scheme 1) and their

Table 1
Chemical shifts of H-2 proton in *cis*- and *trans*-diastereoisomers

Compound	Isomer	^{1}H NMR (ppm)	
		δ(H-2)	$\Delta\delta$[a]
12a	*trans*-	5.38[b]	0.19
12b	*cis*-	5.19[b]	
13a	*trans*-	5.31[b]	0.18
13b	*cis*-	5.13[b]	
13a	*trans*-	5.22[c]	0.27
13b	*cis*-	4.95[c]	
14a	*trans*-	5.31[b]	0.19
14b	*cis*-	5.12[b]	
15a	*trans*-	5.15[c]	0.26
15b	*cis*-	4.89[c]	
16a	*trans*-	5.27[c]	0.29
16b	*cis*-	4.98[c]	
17a	*trans*-	5.19[c]	0.27
17b	*cis*-	4.92[c]	
18a	*trans*-	5.41[b]	0.17
18b	*cis*-	5.24[b]	
18a	*trans*-	5.16[c]	0.27
18b	*cis*-	4.89[c]	
19a	*trans*-	5.36[b]	0.16
19b	*cis*-	5.20[b]	
20a	*trans*-	5.35[c]	0.31
20b	*cis*-	5.04[c]	
21a	*trans*-	5.30[c]	0.29
21b	*cis*-	5.01[c]	
10a[d]	*trans*-	4.44[c]	0.39
10b	*cis*-	4.05[c]	
11a	*trans*-	5.78[c]	0.29
11b	*cis*-	5.49[c]	
8a	*trans*-	4.94[c]	0.32
8b	*cis*-	4.62[c]	

[a] $\Delta\delta = \delta(\text{H-2})_{trans} - \delta(\text{H-2})_{cis}$.
[b] CD_3COCD_3.
[c] $CDCl_3$.
[d] Measured in a mixture containing 25% of *cis*-diastereoisomer.



Product	Nu	Ratio of distereoisomers	
10a, **10b**	CH_3NH	43	57
11a, **11b**	C_6H_5O	32	68
12a, **12b**	C_6H_5NH	51	49
13a, **13b**	4-CH_3-C_6H_4NH	52	48
14a, **14b**	3,4-di-CH_3-C_6H_3NH	50	50
15a, **15b**	4-CH_3O-C_6H_4NH	43	57
16a, **16b**	2-Cl-C_6H_4NH	48	52
17a, **17b**	4-Cl-C_6H_4NH	50	50
18a, **18b**	3,4-di-Cl-C_6H_3NH	57	43
19a, **19b**	4-Br-C_6H_4NH	54	46
20a, **20b**	4-NO_2-C_6H_4NH	58	42
21a, **21b**	4-CF_3-C_6H_4NH	50	50

Scheme 1.

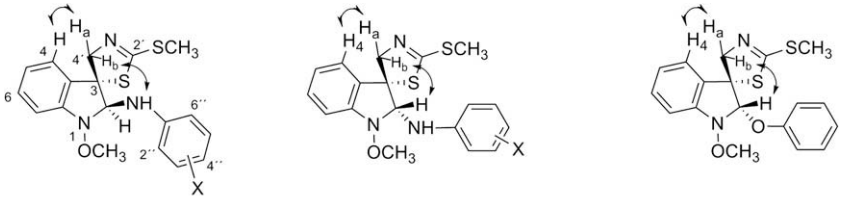


Figure 2. NOESY interactions evidencing the diastereoisomeric structure.

ratio was determined by ^{1}H NMR spectra of crude reaction products obtained after dilution with dichloromethane, washing with 1 M HCl, brine, drying and evaporation of solvent. The ratio of diastereoisomers was determined by integration of separated signals corresponding to the H-2, H_a, H_b, OCH_3 and SCH_3 protons (for protons identification see Fig. 2). The reaction of intermediate **B** with used nucleophiles did not exhibit any diastereoselectivity indicating no difference in steric hindrance for approach of nucleophiles under study from any side of the C=N double bond. Cooling of the reaction mixture up to −20 °C did not change the diastereoselectivity. On the other hand, availability of both diastereoisomers allowed sufficient quantity for examination of biological activity.

Structure of individual diastereoisomers was unequivocally proved by NMR studies, including 2D HSQC, HMBC, and NOESY experiments. In the NOESY spectra of *cis*-diastereoisomers of 2-amino derivatives (Fig. 2) the expected interaction between H_b and H-2 protons, whereas with *trans*-diastereoisomers the interaction between H_b and NH protons was observed (for full details see experimental). 2-Phenoxy derivative **11b** exhibited in its NOESY spectrum the cross peak between H-2 and H_b protons evidencing its *cis*-diastereoisomeric structure (Fig. 2). The spectrum of compound **11a** did not show the interaction between H_b and aromatic protons of phenoxy group, which would confirm its *trans*-configuration. However, the interaction between H-2 and H_b was also not observed and thus the structure of *trans*-distereoisomer was assigned to this product. Figure 3 illustrates the interatomic distances between the above mentioned hydrogens in energy-minimized structures of 2-phenyamino derivatives **12a** (*trans*-) and **12b** (*cis*-).

Inspection of ^{1}H NMR spectra of 2-arylamino derivatives **12a**–**21b** revealed the significant difference in chemical shifts between H-2 protons of *trans*- and *cis*-diastereoisomers. In all cases the δ(H-2)$_{trans}$ appeared at lower field compared to δ(H-2)$_{cis}$ (Table 1). The difference in chemical shifts $\Delta\delta = \delta$(H-2)$_{trans}$ −

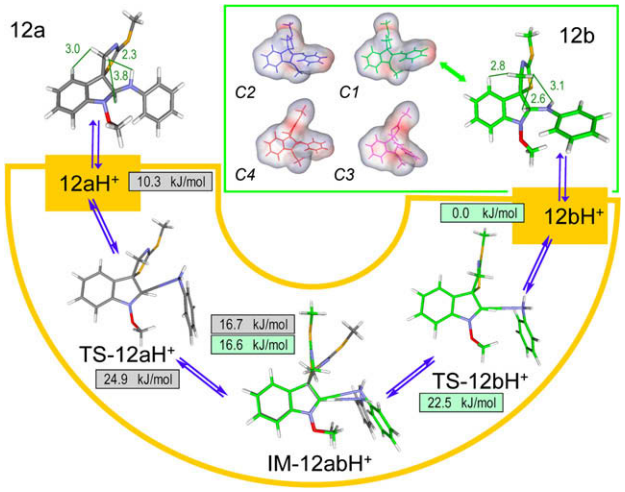
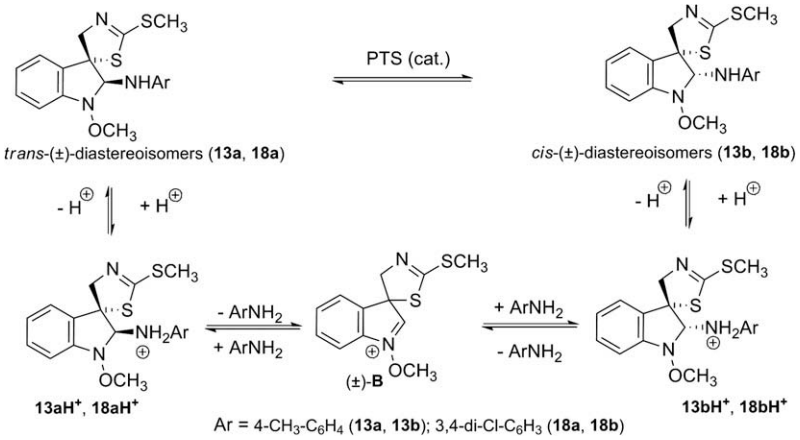


Figure 3. Molecular geometries of **12a** (*trans*; grey carbons) and **12b** (*cis*; green carbons) resulting from DFT B3LYP 6-31** minimizations. The green-boxed part (upper right corner) illustrates the four most stable conformations of **12b** (*C*1 being the lowest-energy form), demonstrating the influence of the thiazoline ring on the molecular shape (showed as semitransparent surfaces). The molecular shape of conformations is influenced also by flipping of the $NOCH_3$ group (compare *C*1 vs *C*2, *C*3 vs *C*4). The conformations and shapes of **12a** and the protonized form of both molecules resembles the shapes of **12b** thus they were not incorporated into the figure. The transition state structures are in the yellow-framed lower part of the figure with superimposition of the energetically close IM forms with their **ΔE** values (in kJ/mol). Selected distances between hydrogen atoms for the most stable conformations of **12a** and **12b** are also indicated.

$\delta(\text{H-2})_{cis}$ falls in the range of 0.16–0.39 ppm. The higher shielding of the H-2 proton in *cis*-diastereoisomers is probably caused by anisotropic shielding imposed by the C=N double bond of the thiazoline moiety. This relation is also valid for 2-methylamino- (**10a**, **10b**), 2-phenoxy- (**11a**, **11b**) as well as natural *trans*-[(±)-**8a**], and unnatural *cis*-[(±)-**8b**] diastereoisomer of 1-methoxyspirobrassinol methyl ether. The chemical shift difference was observed in deuterated chloroform or acetone as demonstrated for 4-methylphenyl (**13a**, **13b**) and 3,4-dichlorophenyl (**18a**, **18b**) derivatives. Consequently the chemical shifts of H-2 protons can be used for assignment of diastereoisomeric structure of 1-methoxyspirobrassinol methyl ether analogs without the study of Nuclear Overhauser Effects (NOE).

Whereas under neutral or basic conditions the synthesized diastereoisomers are stable, during the separation by column chromatography the partial isomerisation of *trans*- to *cis*-isomers was observed and confirmed also on TLC plates when samples of pure diastereoisomers were spotted 10–60 min before developing the plate. In order to study this isomerization quantitatively the 4-methylphenyl (**13a**, **13b**), 3,4-dichlorophenyl (**18a**, **18b**) and 4-nitrophenyl (**20a**, **20b**) derivatives were selected and their separated pure *cis*- and *trans*-isomers dissolved in deuteriochloroform in the presence of 10 mol % of *para*-toluenesulfonic acid (PTS). The isomerization was monitored by ^{1}H NMR spectroscopy at certain periods of time. It was found that both diastereoisomers isomerize and after sufficient time an equilibrium mixture is formed with the *cis*-diastereoisomer prevailing. Both diastereoisomers of 4-methylphenyl derivative (**13a** and **13b**) achieved the equilibrium after 24 h with the ratio *cis*:*trans* = 88:12. In the case of *cis*-3,4-dichlorophenyl derivative **18b** the equilibrium was reached after 3 days (*cis*:*trans* = 68:32), whereas in the equilibrium mixture obtained from the corresponding *trans*-isomer **18a**, the ratio of *cis*:*trans* was 73:27 after three days. With 4-nitrophenyl derivatives **20a** and **20b** the isomerization was slowed down by the electron accepting effect of the nitro group. Equilibrium was not achieved even after three weeks and vast decomposition did not allow completion of the experiment. Isomerization can be of advantage if *cis*-diastereoisomer is required. It can be obtained by isomerization of a crude reaction mixture preceding the chromatographic separation of isomers. Thus if the spirocyclization of 1-methoxybrassinin (**2**) was performed with 2 equiv of 4-methylaniline and the reaction time was prolonged from 5 min to 60 min, the excess of HBr liberated during the reaction caused isomerization, and the *cis*-distereoisomer **13b** was obtained in 65% isolated yield, compared to 42% afforded by standard procedure (see experimental). Analogously, if crude product of a mixture of diastereoisomers **13a** and **13b** was dissolved in dichloromethane and 10 molar % of trifluoroacetic acid was added, *cis*-diastereoisomer **13b** was isolated in 67% yield after 24 h. It is supposed that in acidic medium an amino group can be cleaved off with the formation of 1-methoxyiminium intermediate **B** that subsequently reacts with amine from both sides of C=N double bond with the formation of both isomers (Scheme 2). When equilibrium is achieved, the thermodynamically more stable diastereoisomer prevails.

High-level quantum chemical studies (DFT B3LYP with 6-31** basis set) on all diasteroisomers of 2-amino analogs proved higher stability for the *cis*-isomers than for the *trans*-isomers. Both five-membered heterocycles in the molecules under study exhibited distinct nonplanar conformation, as illustrated in Figure 3 for the model case of **12a** and **12b**. The nonplanar shape is caused by sp^3 characters of the indoline C-2 and C-3 carbons and methoxy substituted nitrogen. The pyramidal inversion of sp^3 nitrogen in the indoline moiety ('up' and 'down' oscillation of the unshared



Scheme 2.

pair and hence the methoxy group as well from one side of the indoline ring to the other) introduces conformational flexibility into these molecules influencing the orientation of the arylamino group. Although the overall molecular shape looks similar for *C*1, *C*2, *C*3 and *C*4 conformations, the shape difference is more evident when comparing the difference in upper (*C*1, *C*2) and lower (*C*3, *C*4) conformations in Figure 3. The influence of the conformational change of the thiazoline ring exhibits significant effect on the molecular shape. The left-turned thiazoline in the *C*1 and *C*2 conformations turns right in the *C*3 and *C*4 conformations. These forms also remain present in the protonized species of the molecules (see Scheme 2 for protonization). The most stable conformation for the *cis*-isomer **12b** is the left-turned *C*1 conformation, whereas the most stable conformation of the *trans*-isomer **12a** (shown in the upper-left corner of Figure 3) has a right-oriented thiazoline ring. The indoline part of the structures becomes fully planar in the transition state of *trans–cis* interconversion. The corresponding molecular structures for the transition state models, resulting from the transition-state guess option of the JAGUAR program[20] with the LST (linear synchronous transit) protocol are in the yellow framed part of Figure 3 together with their relative energies and reaction intermediate (IM-12abH$^+$).

The *cis:trans* (**12b**:**12a**) equilibrium ratio based on all minimized structures resulted 91% in favor of the *cis*-diastereoisomer. The equilibrium of the protonized species was even more shifted towards the *cis*-isomer, approaching 98%. When we reoptimized the molecular geometries including the PBF (Poisson–Boltzmann) solvation model the preference of *cis*-isomer went up by 2% for the neutral and down by 1% for the protonized forms. Solvation calculated in this way has only minor effect on *cis*:*trans* equilibria for the molecules under study.

2.2. Biological evaluation

2.2.1. Antiproliferative/cytotoxic activity

IC_{50} values of newly synthesized compounds and conventional anticancer agents doxorubicin, etoposide and cisplatin on the panel of 6 human cancer cell lines are presented in Table 2. Average potencies of these compounds across all cell lines (expressed as averaged—log IC_{50}), similarity of their fingerprints (growth inhibitory profiles) to that of doxorubicin, etoposide and cisplatin, and *p*-values for statistical significance of the similarity are presented in Table 3.

Among newly synthesized 2-amino analogs of 1-methoxyspirobrassinol methyl ether [(−)-**8**], eight compounds have their average potency higher by at least 50% than compound *trans*-(±)-**8a**, natural diastereoisomer and the more potent of two diastereoisomeric 1-methoxyspirobrassinol methyl ethers *cis*-(±)-**8b** and *trans*-(±)-**8a**. All remaining 2-amino analogs display equal or slightly higher average potency in comparison with compounds *cis*-(±)-**8b** and *trans*-(±)-**8a**, while the other two 2-phenoxy compounds, **11a** and **11b**, were found inactive on our panel. For this reason, synthesis of 2-amino analogs of 1-methoxyspirobrassinol methyl ether seems to be beneficial for anticancer drug design. In addition, comparison of potencies of newly synthesized compounds and conventional anticancer agents demonstrates that

Table 2
Antiproliferative activities of 1-methoxyspirobrassinol methyl ether and its analogs

Compound	R	Isomer	Cell line, IC_{50} (μmol × L^{-1})					
			Jurkat	MCF-7	MDA-MB-231	HeLa	CCRF-CEM	A-549
8a	OCH_3[a]	*trans*-	30.2	100	100	48.9	100	100
8b	OCH_3[a]	*cis*-	57.4	100	100	53.2	100	100
10a	$NHCH_3$	*trans*-	NT[b]	NT	NT	NT	NT	NT
10b	$NHCH_3$	*cis*-	100	100	100	33.6	100	100
11a	OC_6H_5	*trans*-	100	100	100	100	100	100
11b	OC_6H_5	*cis*-	100	100	100	100	100	100
12a	NH–C_6H_5	*trans*-	100	100	100	31.9	100	100
12b	NH–C_6H_5	*cis*-	17.8	100	62.0	25.8	31.8	100
13a	NH-4-CH_3-C_6H_4	*trans*-	33.3	100	100	36.8	100	100
13b	NH-4-CH_3-C_6H_4	*cis*-	6.6	100	78.7	100	64.7	100
14a	NH-3,4-di-CH_3–C_6H_3	*trans*-	47.1	58.0	100	23.8	45.5	42.6
14b	NH-3,4-di-CH_3–C_6H_3	*cis*-	10.0	100	100	54.9	100	100
15a	NH-4-CH_3O–C_6H_4	*trans*-	69.4	100	64.0	100	15.5	100
15b	NH-4-CH_3O–C_6H_4	*cis*-	34.8	100	100	81.5	10.0	73.9
16a	NH-2-Cl–C_6H_4	*trans*-	43.0	100	100	100	75.8	82.0
16b	NH-2-Cl–C_6H_4	*cis*-	29.4	100	100	76.8	36.0	100
17a	NH-4-Cl–C_6H_4	*trans*-	100	28.5	20.7	22.3	100	22.3
17b	NH-4-Cl–C_6H_4	*cis*-	30.7	100	100	44.8	37.4	85.9
18a	NH-3,4-di-Cl–C_6H_3	*trans*-	21.0	22.6	20.7	21.8	22.6	21.1
18b	NH-3,4-di-Cl–C_6H_3	*cis*-	10.0	100	35.5	15.4	100	24.7
19a	NH-4-Br–C_6H_4	*trans*-	33.4	34.1	100	81.5	28.7	35.1
19b	NH-4-Br–C_6H_4	*cis*-	29.3	100	100	55.7	54.8	100
20a	NH-4-NO_2-C_6H_4	*trans*-	100	88.0	100	100	54.5	30.2
20b	NH-4-NO_2-C_6H_4	*cis*-	10.0	100	100	50.0	100	100
21a	NH-4-CF_3–C_6H_4	*trans*-	28.4	66.8	31.1	34.9	54.0	46.6
21b	NH-4-CF_3-C_6H_4	*cis*-	41.8	100	88	81.9	74.3	1.0
Doxorubicin		—	0.078	0.5	0.2	0.2	0.09	1.9
Cisplatin		—	12	11.4	14.7	7.7	4.4	12.2
VP-16 (Etoposide)		—	1.2	10.9	21.2	3.9	1.1	14.3

[a] Synthesized according the published procedure.[12]
[b] The *trans*-diastereoisomer was not tested (NT) since it was obtained only in a mixture containing 25% of *cis*-distereoisomer **10b**.

Table 3
Similarity of growth inhibitory profiles of tested compounds on the panel of 6 cell lines, to the profiles of conventional anticancer agents doxorubicin, etoposide and cisplatin ($-\log IC_{50}$) values were calculated for any given compound across all cell lines; *r*—correlation coefficient of a profile to a selected reference profile; *p*-value—statistical significance of correlation coefficient tested with two-tailed *t*-test (NS: $p > 0.05$)

Compound	$-\log IC_{50}$ ($\mu mol \times L^{-1}$)	Similarity to doxorubicin		Similarity to etoposide		Similarity to cisplatin	
		r	*p*-value	*r*	*p*-value	*r*	*p*-value
Doxorubicin	6.60	1.00	<0.0001	0.74	NS	0.42	NS
Etoposide	5.29	0.74	NS	1.00	<0.0001	0.68	NS
Cisplatin	5.01	0.42	NS	0.68	NS	1.00	<0.0001
18a	4.67	−0.10	NS	−0.33	NS	−0.71	NS
18b	4.48	0.10	NS	0.14	NS	−0.39	NS
21b	4.44	−0.74	NS	−0.28	NS	−0.26	NS
17a	4.42	−0.69	NS	−0.90	0.0145	−0.51	NS
21a	4.38	0.42	NS	0.12	NS	−0.36	NS
12b	4.34	0.84	0.036	0.84	0.0364	0.40	NS
19a	4.34	−0.06	NS	0.48	NS	0.33	NS
14a	4.32	−0.02	NS	0.46	NS	0.49	NS
15b	4.28	0.57	NS	0.83	0.0409	0.79	NS
13b	4.25	0.58	NS	0.62	NS	−0.14	NS
17b	4.23	0.73	NS	0.96	0.0024	0.58	NS
20b	4.22	0.52	NS	0.59	NS	−0.16	NS
14b	4.21	0.52	NS	0.59	NS	−0.17	NS
15a	4.19	0.57	NS	0.59	NS	0.76	NS
16b	4.18	0.77	NS	0.95	0.0037	0.49	NS
19b	4.17	0.75	NS	0.89	0.0175	0.32	NS
13a	4.15	0.47	NS	0.53	NS	0.00	NS
20a	4.14	−0.64	NS	−0.13	NS	0.17	NS
8a	4.14	0.51	NS	0.58	NS	−0.08	NS
16a	4.10	0.45	NS	0.68	NS	−0.01	NS
8b	4.09	0.44	NS	0.49	NS	0.04	NS
12a	4.08	0.10	NS	0.10	NS	0.26	NS
10b	4.08	0.10	NS	0.10	NS	0.26	NS
11a	4.00	0.00	NS	0.00	NS	0.00	NS
11b	4.00	0.00	NS	0.00	NS	0.00	NS

compounds **13b**, **14b**, **18b** and **20b** have potencies higher than or comparable to cisplatin on Jurkat cells; both compounds **17a** and **18a** have higher potencies than etoposide and **18a** has only 1.4 × lower potency than cisplatin on MDA-MB-231 cells; and compound **21b**, the most potent among all tested compounds on A-549 cells, is about 2 × more potent than doxorubicin on these cells. Similarly, the potency of compound **18a** on A-549 cells was found to be comparable to that of etoposide. These results also are promising with regard to the possible future role of these compounds in anticancer drug design and development.

Two-way ANOVA determined that overall variation of ($-\log IC_{50}$) values is caused by cell lines (13.87% of total variation, $p = 0.0002$) and tested compounds (23.98% of total variation, $p = 0.0111$). This finding indicates that cells from our panel respond to tested compounds significantly differently, and that there are significant differences between compounds in their potencies across cell lines. Friedman's test for differences in mean ($-\log IC_{50}$) values among cell lines across all synthesized compounds (not including conventional anticancer agents) also determined significant differences among cell line responses ($p < 0.0001$; Friedman's statistics corrected for ties $F = 35.227$; rows = 25; columns = 6). Dunn's multiple comparisons post test identified significant differences in responses between Jurkat and MCF-7 cells ($p < 0.001$), Jurkat and MDA-MB-231 ($p < 0.01$), Jurkat and A-549 ($p < 0.05$), and HeLa and MCF-7 cells ($p < 0.05$). As a result, statistical analysis demonstrates significantly higher overall sensitivity of Jurkat cells to tested compounds compared to MCF-7, MDA-MB-231, and A-549 cells, which is consistent with generally higher in vitro sensitivity of hemopoietic cells compared to solid tumor cell lines.[21] These differences may result from a number of factors, in addition to the interaction with the target involved in the cytostatic/cytotoxic response. These factors include number of cell cycles during the incubation period, drug uptake, efflux and metabolism.[22] On the other hand, few differences among cell lines would suggest, that some non-specific cytotoxic effect is responsible for the activity of tested compounds.

Results of Fisher's exact test demonstrate statistically significant association between *cis*:*trans* diastereoisomeric status of tested compounds and their cytostatic/cytotoxic effects for MCF-7 breast cancer cell line ($p = 0.0052$), but not for other employed cell lines ($p > 0.05$). In MCF-7 cells, all *cis*-diastereoisomers were classified as inactive ($IC_{50} > 90$ $\mu mol \times L^{-1}$), while 6 out of 12 *trans*-diastereoisomers demonstrated potency with $IC_{50} < 90$ $\mu mol \times L^{-1}$. In contrast, a similar association was not found for MDA-MB-231 cells that were also derived from human breast adenocarcinoma and have approximately the same overall chemosensitivity as MCF-7 cells (average $-\log IC_{50}$ for all tested compounds except for doxorubicin, etoposide and cisplatin on MCF-7 and MDA-MB-231 cells is 4.09 and 4.12, respectively). The association between *cis:trans* stereochemistry and activity classification on MCF-7 cells remains to be elucidated, and we speculate a possible role for estrogen receptors that are present in MCF-7, but not in MDA-MB-231 cells.

Growth inhibitory profiles (fingerprints) of compounds **12b**, **15b**, **16b**, **17b** and **19b** against the panel of 6 cell lines are strongly positively, and statistically significantly correlated to the profile of etoposide (Table 3), an anticancer agent that acts via inhibition of topoisomerase II.[23] In general, growth inhibitory profiles of drugs against diverse cancer cell lines correlate well with their mode of action;[24] therefore, our results suggest, that compounds **12b**, **15b**, **16b**, **17b** and **19b** likely inhibit topoisomerase II as a part of their anticancer mode of action. Interestingly, the profile of compound **17a**, a diastereoisomer of compound **17b**, is strongly negatively and significantly correlated with that of etoposide (Fig. 4), which indicates a different mode of action for **17a**. Thus, the analysis of fingerprints of the tested compounds reveals the possibility of at least two different anticancer modes of action within the set of synthesized 2-amino analogs of 1-methoxyspiro-

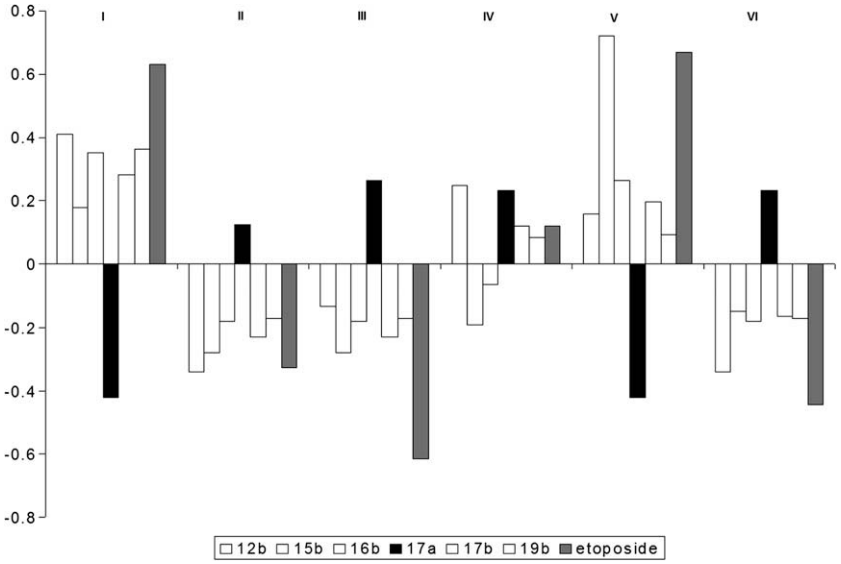


Figure 4. Growth inhibitory profiles of compounds **12b**, **15b**, **16b**, **17b**, and **19b** (empty bars), etoposide (gray bars) and **17a** (black bars). Cell lines: I—Jurkat; II—MCF-7; III—MDA-MB-231; IV—HeLa; V—CCRF-CEM; VI—A-549. Vertical axis: ($-\log IC_{50}$) of a compound on a given cell line—averaged ($-\log IC_{50}$) of that compound across all cell lines.

brassinol methyl ether. In addition, our data demonstrate strong positive and statistically significant correlation of compound **12b** profile with that of doxorubicin, whose mode of action also includes inhibition of topoisomerase II.[25] There were no statistically significant correlations between the profiles of tested compounds and cisplatin.

Hierarchical clustering applied to our chemosensitivity data is presented in the form of a heat map with red, green and yellow color for high, low and intermediate potency of a given compound and cell line compared to the average potency of that compound across all cell lines (Fig. 5). Drugs with the same modes of action or targets form clusters in this type of clustering[26] and, consistent with the profile correlation analysis, our results display clustering of compounds **12b**, **16b**, **17b,** etoposide, and **19b**. Overall, 3 large clusters can be identified from the dendrogram: one cluster that includes inactive compounds **11a** and **11b**, a second that contains compounds **15a**, **15b**, **19a**, **20a** and **21b**, and a third with all remaining compounds. However, due to the complex pattern of clustering within the third cluster, it is likely that it also includes compounds that differ in their anticancer mode of action. Clustering of cell types is consistent with cell origin, and shows clear separation of leukemias from solid tumors and clustering of MCF-7 and MDA-MB-231 breast cancer cell lines.

2.2.2. Antimicrobial activity

Synthesized compounds expressed mild antibacterial activity against selected bacterial strains. The most valuable finding is the inhibition of *Streptococcus pyogenes* growth by compound

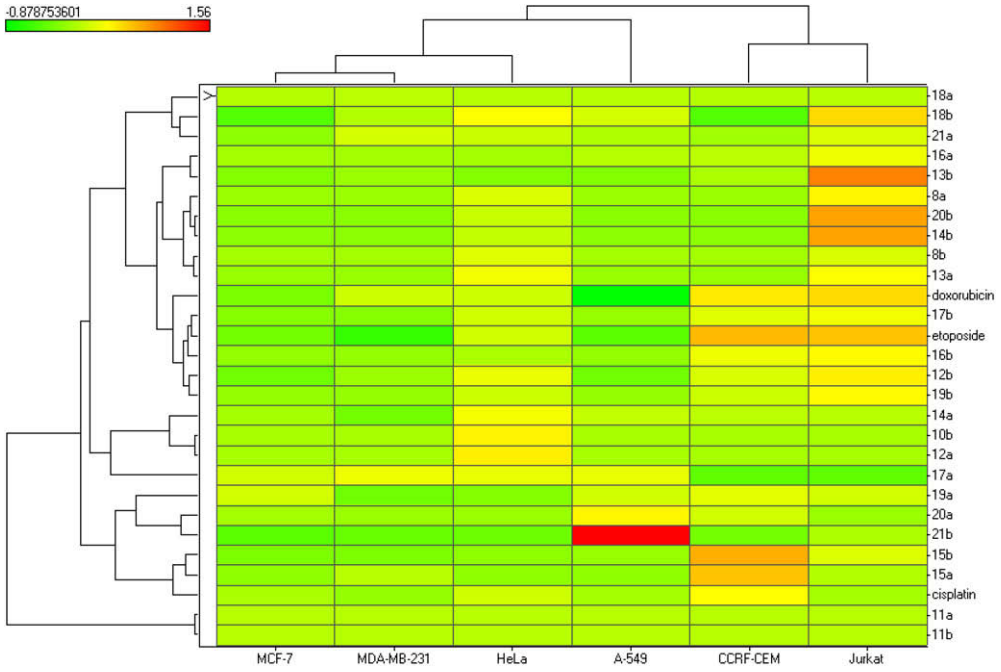


Figure 5. Heat map with hierarchical clustering for ($-\log IC_{50}$)—averaged ($-\log IC_{50}$) for a given compound across all cell lines. Values are visualized by color scheme with red color for high, yellow color for intermediate and green color for low values.

19b. In spite of its relatively low potency (diameter of inhibition zone was 7 mm, diameter of the disc was 6 mm), the zone of inhibition was clear and remained stable over the next two days. Similarly, compounds **18a**, **21a**, **21b** showed inhibitory activity against *Bacillus subtilis*; however, the potencies of tested compounds were also relatively low. Activity of tested compounds appears to be directed against gram positive bacteria. Among all, the most potent compound was **19b** active against *S. pyogenes*. Compound **19b** could potentially serve as lead molecule for synthesis of more effective agents against *S. pyogenes*, bacteria that causes suppurative infections with sometimes serious and lethal complications, including necrotizing fasciitis and streptococcal toxic shock-like syndrome (STSS).

2.3. Structure–activity relationships

The Phase program of Schrodinger Inc.[27] was used for pharmacophore modeling and 3D QSAR analysis of the studied compounds. Although Phase is a relatively new method,[28] according to Evans and coworkers,[29] it performed comparably or even better than the well-established Catalyst HypoGen for their tested set of compounds. We were aimed accordingly in Phase in order to generate pharmacophores for molecules synthesized in our laboratories. The DFT B3LYP 6-31** minimized structures were used as input geometries for Phase to generate conformations and create hypotheses according to activities in the second step. Hypotheses were then scored, clustered and visually analyzed with active molecules superposed. 3D QSAR models were then generated for the most predictive hypotheses.

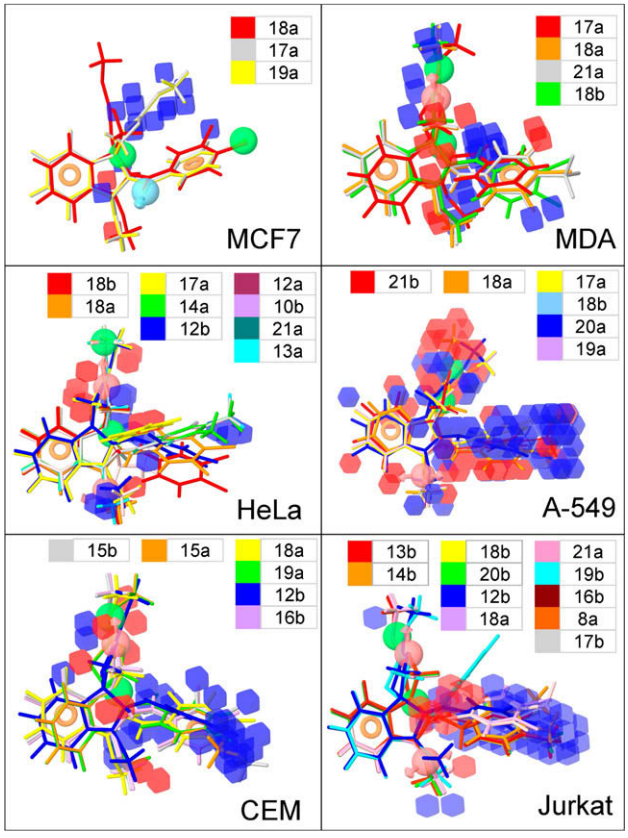


Figure 6. Pharmacophore (pink circles correspond to proton-acceptor, light blue to proton donor, green to hydrophobic groups and orange rings to aromatic fragments) and 3D QSAR models (blue and red cubes) projected onto the superposed most active compounds for the different cell lines. Blue cubes for the QSAR models represent positive contribution to activity increase; red cubes determine negative contribution decreasing the activity. The most active compounds were superimposed into the given pharmacophore models and their color-coding is shown individually for each cell line. Cell lines: MCF7; MDA-MB-231 (MDA); HeLa; A-549; CCRF-CEM (CEM); Jurkat.

Figure 6 illustrates such models for all cell lines under study. The blue cubes represent positive contribution to the activity whereas the red cubes represent negative contribution decreasing the activities. The presented models can be clustered into two groups. In the first group (Jurkat, CCRF-CEM and A-549) the substituted phenyl ring has a significant impact on molecular activity, assuming the Jurkat-related molecules being best aligned and exhibiting simultaneously the best activities. These results reasonably correlate with the right part of Figure 5 illustrating heat map clustering. The most active molecules for Jurkat have *cis*-configuration with left oriented thiazoline ring. For CCRF-CEM model, both the *cis*- and *trans*-isomers are equally present between the superposed molecules and the thiazoline fragment exhibits both the left and the right orientation. The negative contribution of thiazoline to the inhibition is still insignificant according to the number of red boxes shown in this part of the molecule. This scenario is changed for A-549 where only two active molecules are in *cis*-configuration and the majority of the active analogs are *trans*-isomers. In this cell line, the importance of the thiazoline fragment is increasing in both a positive, as well as a negative way.

The second group of cell lines (MDA-MB-231, HeLa, MCF-7) and the molecules do not exhibit equivalent importance for the substitution on the phenylamino moiety. The structures of investigated compounds do not superpose so effectively and the MCF-7 3D QSAR model is the only one where the right-oriented thiazoline group might have a dominant effect on growth inhibition. Interestingly, the protonization studies indicated that the thiazoline ring might undergo conformational change from left to right orientation if the ammonium group is present in the molecule. According to the 3D QSAR model, the substitution of the arylamino group is equally disturbing as activity supporting for A-549, while for MDA-MB-231 and MCF-7 cells the thiazoline fragment becomes the most significant part of the molecules improving their activity profile. The thiazoline part corresponds to the pharmacophore for hydrophobic interactions and is probably involved in binding. Despite the presence of a nitrogen atom in thiazoline ring, the hydrogen-bond acceptor is not a part of the pharmacophore for MCF-7 cells. This might be the reason why the studied molecules provide the weakest activity profile for the MCF-7 cell line in accordance with Figure 5.

3. Conclusion

Analogs of indole phytoalexin 1-methoxyspirobrassinol methyl ether [(−)-**8**] that possess the substituted phenyl amino group instead of the methoxy group in position 2 of indoline moiety have been designed and synthesized as new prospective anticancer compounds. Using the effective synthetic strategy based on tandem electrophilic–nucleophilic 3,2-heterodifunctionalisation of indole and following the Topliss operational scheme, a set of *cis*- and *trans*-diastereoisomers of target compounds was synthesized. We found that *cis*-isomers were thermodynamically more stable than *trans*-isomers, which can be isomerized to the corresponding *cis*-diastereoisomers in acidic medium. The observed significant differences in chemical shifts of the H-2 proton between *cis*- and *trans*-diastereoisomers in ^{1}H NMR spectra can be used for the reliable assignment of the diastereoisomeric structure of new compounds without performing the NOE experiments.

We examined cytostatic/cytotoxic activity of synthesized compounds against selected human solid tumor and leukemia cell lines in vitro (Jurkat, MCF-7, MDA-MB-231, HeLa, CCRF-CEM and A-549). Compounds **13b**, **14b**, **18b** and **20b** have potencies higher than or comparable to cisplatin on Jurkat cells, compounds **17a** and **18a**

have higher potencies than etoposide on MDA-MB-231 cells, and compound **21b**, that was found to be the most potent among all tested compounds on A-549 cells, is about 2 × more potent than doxorubicin on A-549 cells. These results are promising with regard to the possible future role of these compounds in anticancer drug development. The pharmacophore/3D QSAR modeling highlighted the molecular fragments of the lead molecules responsible for activities on different cell lines. More targeted molecular design on selected lead molecules taking into account all experience coming from the 3D/pharmacophore modeling will be the subject of our future studies.

4. Experimental

4.1. Chemistry

Melting points were determined on a Koffler micro melting point apparatus and are uncorrected. IR spectra were recorded on an IR-75 spectrometer (Zeiss Jena). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured on a Varian Mercury Plus spectrometer. Chemical shifts (δ) are reported in ppm downfield from TMS as internal standard and coupling constants (J) are given in hertz. Microanalyses were performed with a Perkin–Elmer, Model 2400 analyzer. The EI mass spectra were recorded on a GC–MS Trio 1000 (Fisons Instruments) spectrometer at ionization energy of 70 eV, whereas MALDI-TOF mass spectra of **10b**, **17a** and **17b** were measured on a MALDI IV (Shimadzu, Kratos Analytical). The samples were ionized with a N_2-laser (λ = 337 nm). The progress of chemical reactions was monitored by thin layer chromatography, using Macherey-Nagel plates Alugram®Sil G/UV254. Preparative column chromatography was performed on Kieselgel 60 Merck Type 9385 (0.040–0.063 mm) and aluminum oxide 90 neutral, Merck (0.063–0.200 mm, activity I).

4.1.1. Improved procedure for the preparation of 1-methoxybrassinin (2)

Crude product of (1-methoxyindole-3-yl)methylamine freshly prepared by the reduction of 1-methoxyindole-3-caboxaldehyde oxime (0.570 g, 3 mmol) with $NaBH_3CN$ in the presence of $TiCl_3$[18] was dissolved in methanol (24 mL). Triethylamine (1.25 mL, 0.91 g, 9 mmol) and carbon disulfide (0.54 mL, 0.685 g, 9 mmol) were added and mixture was stirred for 5 min at room temperature. Then iodomethane (0.56 mL, 1.277 g, 9 mmol) was added and stirring was continued for 15 min. The residue obtained after evaporation of the solvent was dissolved in dichloromethane, small amount of silica gel was added, dichloromethane evaporated and product preabsorbed on silica gel was chromatographed on silica gel (45 g, hexane–acetone 5:1). Yield 0.61 g, 76%. Spectral data of the obtained 1-methoxybrassinin (**2**) were identical with those described previously.[18]

4.1.2. *trans*- and *cis*-1-Methoxy-2-methylamino-2′-(methylsulfanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazoles} (10a and 10b)

To a stirred solution of 1-methoxybrassinin (**2**, 0.1 g, 0.375 mmol) with powder molecular sieves (3 Å) in anhydrous dichloromethane (4 mL) at room temperature was added freshly prepared solution of bromine (0.96 mL, 0.413 mmol, stock solution prepared by dissolving of 0.04 mL of Br_2 in 1.76 mL of anhydrous dichloromethane). After stirring for 5 min, a mixture of methylamine hydrochloride (0.051 g, 0.75 mmol, dried at 75 °C for 90 min) and triethylamine (0.721 g, 1.0 mL, 7.125 mmol) in anhydrous dichloromethane (5 mL) was added. After being stirred for 5 min, the reaction mixture was diluted with dichloromethane (10 mL), successively washed with 1 M HCl (10 mL), water (20 mL), and brine (20 mL), and dried over Na_2SO_4. The residue obtained after solvent removal in vacuum was submitted to chromatography on aluminum oxide 90 neutral, 0.063–0.200 mm, activity I, Merck (15 g, diethyl ether/*n*-hexane 1:4) to give products **10a** (0.013 g, 12%, containing 25% of **10b** as inseparable impurity) and **10b** (0.029 g, 26%). *trans-1-Methoxy-2-methylamino-2′-(methylsulfanyl)spiro{indoline-3,5′-[4′,5′]dihydrothiazole}* (**10a**). Colorless oil, R_f = 0.11 (diethyl ether/*n*-hexane, 1:4). ^{1}H NMR ($CDCl_3$) δ 2.55 (broad singlet, 1H, NH), 2.56 (s, 3H, SCH_3), 2.69 (s, 3H, N–CH_3), 3.90 (d, J = 15.5 Hz, 1H, H-4′), 3.93 (s, 3H, OCH_3), 4.44 (s, 1H, H-2), 4.73 (d, J = 15.5 Hz, 1H, H-4′), 6.95 (d, J = 7.9 Hz, 1H, ArH), 7.01 (ddd, J = 1.0, 7.5, 7.5 Hz, 1H, ArH), 7.24 (ddd, J = 1.2, 7.6, 7.6 Hz, 1H, ArH), 7.30 (dd, J = 1.1, 7.5 Hz, 1H, ArH). ^{13}C NMR ($CDCl_3$) δ 15.3, 35.3, 64.1, 69.5, 69.8, 94.8, 113.0, 123.8, 123.9, 129.6, 129.8, 149.7, 165.4. *cis-1-Methoxy-2-methylamino-2′-(methylsulfanyl)-spiro{indoline-3,5′-[4′,5′]dihydrothiazole}* (**10b**). Colorless oil, R_f = 0.19 (diethyl ether/*n*-hexane, 1:4). IR ($CHCl_3$) ν_{max} 3573, 3100, 2307, 1700, 1393, 1113, 980, 787 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 2.39 (broad singlet, 1H, NH), 2.52 (s, 3H, SCH_3), 2.63 (broad singlet, 3H, N–CH_3), 3.86 (s, 3H, OCH_3), 4.08 (broad singlet, 1H, H-2), 4.27 (d, J = 15.7 Hz, 1H, H-4′$_b$), 4.61 (d, J = 15.7 Hz, 1H, H-4′$_a$), 6.94 (d, J = 7.6 Hz, 1H, H-7), 7.00 (dd, J = 7.3, 7.4 Hz, 1H, H-5), 7.22–7.27 (m, 2H, H-4, H-6). ^{13}C NMR (DMSO-d_6) δ 14.5 (SCH_3), 34.1 (NCH_3), 63.1 (OCH_3), 70.5 (C-3), 72.7 (C-4′), 91.6 (C-2), 112.2 (C-7), 122.7 (C-4), 123.2 (C-5), 129.3 (C-6), 129.7 (C-3a), 149.1 (C-7a), 161.7 (C-2′). NOESY correlations: N–CH_3/H-2; NH/N–CH_3; H-2/H-4′$_b$, N–CH_3; H-4′$_b$/H-2, H-4′$_a$; H-4′$_a$/H-4′$_b$, H-4; H-7/H-6; H-5/H-4, H-6; H-4/H-4′$_a$, H-5; H-6/H-5, H-7. MALDI-TOF MS, *m/z* (relative intensity): 295.3 M$^+$ (84%), 264.7 (100%), 199.3 (26%), 160.7 (24%). Anal. Calcd for $C_{13}H_{17}N_3OS_2$ requires: C, 52.85; H, 5.80; N, 14.22. Found: C, 52.71; H, 5.59; N, 14.02.

4.1.3. *trans*- and *cis*-1-Methoxy-2-phenoxy-2′-(methylsulfanyl)-spiro{indoline-3,5′-[4′,5′]dihydrotiazoles} (11a and 11b)

To a stirred solution of 1-methoxybrassinin (**2,** 0.08 g, 0.3 mmol) with powdered molecular sieves (3Å) in anhydrous dichloromethane (5 mL) was added freshly prepared solution of bromine (0.76 mL, 0.33 mmol, stock solution prepared by dissolving of 0.04 mL of Br_2 in 1.76 mL of anhydrous dichloromethane). After stirring for 5 min, the solution of phenol (0.056 g, 0.6 mmol), triethylamine (0.306 g, 0.421 mL, 3 mmol) in dry dichloromethane (5 mL) with powdered molecular sieves (3 Å) was added. Stirring was continued for another 20 min, the reaction mixture diluted with dichloromethane (25 mL), washed with 1 M HCl (6 mL) and brine (15 mL). Organic layer was dried over anhydrous Na_2SO_4 and the residue obtained after evaporation of solvent was subjected to chromatography on silica gel (23 g, hexane/acetone 5:1), affording the products **11a** (0.022 g, 20%) and **11b** (0.073 g, 68%). *trans*-1-Methoxy-2-phenoxy-2′-(methylsulfanyl)spiro{indoline-3,5′-[4′,5′]dihydrothiazole} (**11a**). Colorless oil; R_f = 0.45 (hexane/acetone, 5:1). ^{1}H NMR ($CDCl_3$) δ 2.52 (s, 3H, SCH_3), 3.87 (s, 3H, OCH_3), 4.01 (d, J = 15.4 Hz, 1H, H-4′$_a$), 5.18 (d, J = 15.4 Hz, 1H, H-4′$_b$), 5.78 (s, 1H, H-2), 6.99 (dd, J = 7.8, 0.8 Hz, 1H, H-7), 7.06 (ddd, J = 1.1, 7.6 Hz, 1H, H-5), 7.06 (dddd, J = 1.1, 1.2, 7.3, 7.3 Hz, 1H, H-4″), 7.18 (dd, J = 1.1, 8.7 Hz, 2H, H-2″, H-6″), 7.29 (ddd, J = 1.2, 7.7, 7.7 Hz, 1H, H-6), 7.32 (dd, J = 7.3, 8.6 Hz, 2H, H-3″, H-5″), 7.35 (dd, J = 1.2, 7.6 Hz, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ 15.1 (SCH_3), 64.0 (OCH_3), 69.3 (C-3), 70.2 (C-4′), 107.3 (C-2), 113.0 (C-7), 117.8 (C-2″,C-6″), 122.9 (C-4″), 123.9 (C-4), 124.0 (C-5), 127.0 (C-3a), 129.6 (C-3″, C-5″), 129.9 (C-6), 147.3 (C-7a), 158.2 (C-1″), 163.5 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_a$/H-4′$_b$, H-4; H-4′$_b$/H-4′$_a$; H-2/H-2″, H-6″; H-7/OCH_3, H-6; H-5/H-6, H-4; H-4″/H-3″, H-5″; H-2″, H-6″/H-2, H-3″, H-5″; H-6/H-7, H-5; H-3″, H-5″/H-4″, H-2″, H-6″; H-4/H-4′$_a$, H-5. EIMS *m/z* (relative intensity): 359 M$^+$ (5%), 265 (100%), 251 (76%), 161 (35%), 160 (25%), 117 (52%). Anal. Calcd for $C_{18}H_{18}N_2O_2S_2$ requires: C, 60.31; H, 5.06; N, 7.81. Found: C, 60.03; H, 5.40; N, 7.53. *cis-1-Methoxy-2-phenoxy-2′-(methylsulfanyl)spiro{indoline-3,5′-[4′,5′]dihydrothiazole}* (**11b**). Colorless oil;

R_f = 0,35 (hexane/acetone, 5:1). ^{1}H NMR ($CDCl_3$) δ 2.54 (s, 3H, SCH_3), 3.88 (s, 3H, OCH_3), 4.31 (d, J = 15.3 Hz, 1H, H-4′$_b$), 4.44 (d, J = 15.3 Hz, 1H, H-4′$_a$), 5.49 (s, 1H, H-2), 7.00 (dd, J = 1.0, 8.1 Hz, 1H, H-7), 7.06 (ddd, J = 1.0, 7.6, 7.6 Hz, 1H, H-5), 7.07 (dddd, J = 1.1, 1.1, 7.3, 7.3 Hz, 1H, H-4″), 7.18 (dd J = 1.0, 8.7 Hz, 2H, H-2″, H-6″), 7.31 (dd, J = 1.2, 7.4 Hz, 1H, H-4), 7.31 (ddd, J = 1.1, 7.6, 8.0 Hz, 1H, H-6), 7.32 (dd, J = 7.3, 8.7 Hz, 1H, H-3″, H-5″). ^{13}C NMR ($CDCl_3$) δ 15.1 (SCH_3), 63.9 (OCH_3), 70.7 (C-3), 72.6 (C-4′), 103.3 (C-2), 112.7 (C-7), 118.0 (C-2″, C-6″), 123.1 (C-4″), 123.2 (C-4), 124.0 (C-5), 127.8 (C-3a), 129.6 (C-3″, C-5″), 130.0 (C-6), 147.5 (C-7a), 158.3 (C-1″), 166.31 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_a$/H-4′$_b$, H-4; H-4′$_b$/H-4′$_a$, H-2; H-2/H-4′$_b$, H-2″, H-6″; H-7/OCH_3, H-6; H-5/H-6, H-4; H-4″/H-3″, H-5″; H-2″, H-6″/H-2, H-3″, H-5″; H-4/H-4′$_a$, H-5; H-6/H-7, H-5; H-3″, H-5″/H-4″, H-2″, H-6″. EIMS m/z (relative intensity): 359 M$^+$ (5%), 265 (100%), 251 (76%), 161 (35%), 160 (25%), 117 (52%). Anal. Calcd for $C_{18}H_{18}N_2O_2S_2$ requires: C, 60.31; H, 5.06; N, 7.81. Found: C, 60.60; H, 4.88; N, 8.05.

4.1.4. General procedure for the synthesis of *trans*- and *cis*-diastereoisomers of 2-(substituted phenyl)amino analogs of 1-methoxyspirobrassinol methyl ether (12a–21a and 12b–21b)

To a stirred solution of 1-methoxybrassinin (**2**, 0.1 g, 0.375 mmol) in anhydrous dichloromethane (4 mL) at room temperature was added freshly prepared solution of bromine (0.96 mL, 0.413 mmol, stock solution prepared by dissolving of 0.04 mL of Br_2 in 1.76 mL of anhydrous dichloromethane). After stirring for 5 min, a solution of corresponding substituted aniline (0.75–3.375 mmol) and triethylamine (0.721 g, 1.0 mL, 7.125 mmol) in anhydrous dichloromethane (5 mL) was added. After being stirred for 5 min, the reaction mixture was diluted with dichloromethane (10 mL), successively washed with 1 M HCl (10 mL), water (20 mL), and brine (20 mL), and dried over Na_2SO_4. The residue obtained after solvent removal in vacuum was submitted to chromatography on silica gel to give products **12a**–**21a** and **12b**–**21b**.

4.1.4.1. *trans*- and *cis*-1-Methoxy-2-phenylamino-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (12a and 12b). Following the general procedure products **12a** and **12b** were obtained using 0.314 g (0.31 mL, 3.375 mmol) of aniline and separated on silica gel (20 g, diethyl ether/*n*-hexane 1:3). *trans-1-Methoxy-2-phenylamino-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**12a**). Yield: 0.030 g (22%); colorless crystals; mp 131–132 °C (acetone–hexane); R_f = 0.14 (diethyl ether/*n*-hexane, 1:3). IR ($CHCl_3$) ν_{max} 3410, 3010, 2930, 1600, 1400, 1100, 990, 940, 620 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 2.47 (s, 3H, SCH_3), 3.79 (s, 3H, OCH_3), 3.89 (d, J = 15.6 Hz, 1H, H-4′$_a$), 4.92 (d, J = 15.6 Hz, 1H, H-4′$_b$), 5.38 (d, J = 10.2 Hz, 1H, H-2), 5.64 (d, J = 10.2 Hz, 1H, NH), 6.71 (dd, J = 7.3, 7.3 Hz, 1H, H-4″), 6.97 (d, J = 7.9 Hz, 2H, H-2″,H-6″), 6.99 (d, J = 7.7 Hz, 1H, H-7), 7.06 (dd, J = 7.5, 7.6 Hz, 1H, H-5), 7.15 (dd, J = 7.3, 8.0 Hz, 2H, H-3″, H-5″), 7.29 (dd, J = 7.6, 7.7 Hz, 1H, H-6), 7.35 (d, J = 7.5 Hz, 1H, H-4). ^{13}C NMR (acetone-d_6) δ 14.9 (SCH_3), 64.2 (OCH_3), 70.5 (C-3), 70.9 (C-4′), 90.2 (C-2), 114.1 (C-7), 115.2 (C-2″, C-6″), 119.1 (C-4″), 124.4 (C-5), 124.6 (C-4), 129.0 (C-3a), 129.8 (C-3″, C-5″), 130.3 (C-6), 148.5 (C-1″), 150.0 (C-7a), 163.7 (C-2′). NOESY correlations: H-4′$_a$/H-4′$_b$; H-4′$_b$/H-4′$_a$, NH; H-2/NH, H-2″, H-6″; NH/H-4′$_b$, H-2, H-2″, H-6″; H-4″/H-2″, H-6″, H-3″, H-5″; H-2″, H-6″/H-2, NH, H-4″, H-3″, H-5″; H-5/H-6; H-3″, H-5″/H-4″, H-2″, H-6″; H-6/H-5. EIMS m/z (relative intensity): 358 M$^+$ (1%), 326 (100%), 265 (6%), 233 (13%), 161 (18%), 117 (17%), 104 (26%), 77 (68%) Anal. Calcd for $C_{18}H_{19}N_3OS_2$ requires: C, 60.47; H, 5.36; N, 11.75. Found: C, 60.29; H, 5.48; N, 11.93. *cis-1-Methoxy-2-phenylamino-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**12b**). Yield: 0.062 g (46%); yellow oil; R_f = 0.37 (diethyl ether/*n*-hexane, 1:3). IR ($CHCl_3$) ν_{max} 3400, 2927, 1700, 1593, 1460, 1400, 1120, 987, 933, 733, 573 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 2.51 (s, 3H, SCH_3), 3.72 (s, 3H, OCH_3), 4.33 (d, J = 15.8 Hz, 1H, H-4′$_b$), 4.72 (d, J = 15.8 Hz, 1H, H-4′$_a$), 5.19 (d, J = 11.0 Hz, 1H, H-2), 5.32 (d, J = 11.0 Hz, 1H, NH), 6.73 (dddd, J = 1.1, 1.1, 7.4, 7.4 Hz, 1H, H-4″), 6.98 (dd, J = 1.1, 8.3 Hz, 1H, H-7), 7.05 (dd, J = 1.1, 8.6 Hz, 2H, H-2″, H-6″), 7.06 (ddd, J = 1.1, 7.4, 7.6 Hz, 1H, H-5), 7.17 (dd, J = 7.3, 8.6 Hz, 2H, H-3″, H-5″), 7.30 (ddd, J = 1.2, 7.4, 7.5 Hz, 1H, H-6), 7.31 (d, J = 7.5 Hz, 1H, H-4). ^{13}C NMR (acetone-d_6) δ 15.0 (SCH_3), 64.2 (OCH_3), 72.1 (C-3), 73.4 (C-4′), 86.4 (C-2), 113.8 (C-7), 115.3 (C-2″, C-6″), 119.4 (C-4″), 123.6 (C-4), 124.5 (C-5), 129.9 (C-3″, C-5″), 130.3 (C-3a), 130.5 (C-6), 147.7 (C-1″), 149.8 (C-7a), 163.0 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; H-2/H-4′$_b$, NH, H-2″, H-6″; NH/H-2, H-2″, H-6″; H-4″/H-3″, H-5″; H-7/OCH_3, H-6; H-2″, H-6″/H-2, NH, H-3″, H-5″; H-5/H-6, H-4; H-3″, H-5″/H-4″, H-2″, H-6″; H-6/H-7, H-5; H-4/H-4′$_a$, H-5. EIMS m/z (relative intensity) 358 M$^+$ (1%), 326 (100%), 265 (12%), 233 (12%), 161 (20%), 104 (26%), 77 (47%). MALDI-TOF MS, m/z (relative intensity): 396.7 [M+K]$^+$ (9%), 380.7 [M+Na]$^+$ (20%), 357.9 [M+H]$^+$ (16%), 326.4 (50%), 283.3 (26%), 265.8 (100%), 222.2 (42%), 171.5 (33%), 161.6 (28%), 108.3 (50%). Anal. Calcd for $C_{18}H_{19}N_3OS_2$ requires: C, 60.47; H, 5.36; N, 11.75. Found: C, 60.71; H, 5.65; N, 11.99.

4.1.4.2. *trans*- and *cis*-1-Methoxy-2-(4-methylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (13a and 13b). Following the general procedure products **13a** and **13b** were obtained using 0.362 g (3.375 mmol) of 4-methylaniline and separated on silica gel (20 g, ethyl acetate/*n*-hexane 1:8). *trans-1-Methoxy-2-(4-methylphenylamino)-2′-(methylsulphanyl)-spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**13a**). Yield: 0.035 g (25%); colorless crystals, mp 155–157 °C (acetone–hexane); R_f = 0.18 (ethyl acetate/*n*-hexane, 1:8). IR ($CHCl_3$) ν_{max} 3420, 3010, 1610, 1560, 1510, 1460, 1400, 1290, 1105, 995, 960, 805, 620 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 2.20 (s, 3H, CH_3), 2.48 (s, 3H, SCH_3), 3.78 (s, 3H, OCH_3), 3.88 (d, J = 15.6 Hz, 1H, H-4′$_a$), 4.91 (d, J = 15.6 Hz, 1H, H-4′$_b$), 5.31 (d, J = 10.2 Hz, 1H, H-2), 5.45 (d, J = 10.2 Hz, 1H, NH), 6.87 (d, J = 8.4 Hz, 2H, H-2″, H-6″), 6.97 (d, J = 8.4 Hz, 2H, H-3″, H-5″), 6.99 (d, J = 7.8 Hz, 1H, H-7), 7.05 (ddd, J = 1.0, 7.5, 7.5 Hz, 1H, H-5), 7.28 (ddd, J = 1.2, 7.6, 7.8 Hz, 1H, H-6), 7.34 (dd, J = 1.1, 7.6 Hz, 1H, H-4). ^{13}C NMR (acetone-d_6) δ 15.0 (SCH_3), 20.5 (CH_3), 64.2 (OCH_3), 70.5 (C-3), 70.9 (C-4′), 90.7 (C-2), 114.0 (C-7), 115.4 (C-2″, C-6″), 124.4 (C-5), 124.6 (C-4), 127.9 (C-4″), 129.0 (C-3a), 130.3 (C-6), 130.3 (C-3″, C-5″), 146.2 (C-1″), 150.1 (C-7a), 163.6 (C-2′). NOESY correlations: CH_3/H-3″, H-5″; H-4′$_a$/H-4′$_b$, H-4; H-4′$_b$/H-4′$_a$, NH; H-2/NH, H-2″, H-6″; NH/H-4′$_b$, H-2, H-2″, H-6″; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-3″, H-5″/CH_3, H-2″, H-6″; H-7/H-6; H-5/H-6, H-4; H-6/H-7, H-5; H-4/H-5. EIMS m/z (relative intensity) 372 M$^+$ (1%), 340 (100%), 265 (22%), 233 (24%), 161 (25%), 117 (18%), 91 (32%). Anal. Calcd for $C_{19}H_{21}N_3OS_2$ requires: C, 61.42; H, 5.70; N, 11.31. Found: C, 61.73; H, 5.47; N, 11.56. *cis-1-Methoxy-2-(4-methylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**13b**). Yield: 0.059 g (42%); colorless oil; R_f = 0.38 (ethyl acetate/*n*-hexane, 1:8). IR ($CHCl_3$) ν_{max} 3400, 3010, 2930, 1605, 1570, 1510, 1460, 1400, 1240, 1095, 1040, 985, 940, 805, 610 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, CH_3), 2.53 (s, 3H, SCH_3), 3.75 (s, 3H, OCH_3), 4.22 (d, J = 15.6 Hz, 1H, H-4′$_b$), 4.66 (d, J = 10.6 Hz, 1H, NH), 4.68 (d, J = 15.6 Hz, 1H, H-4′$_a$), 4.95 (d, J = 10.6 Hz, 1H, H-2), 6.83 (d, J = 8.2 Hz, 2H, H-2″, H-6″), 6.96 (dd, J = 0.6, 7.8 Hz, 1H, H-7), 7.01 (d, J = 8.4 Hz, 2H, H-3″, H-5″), 7.03 (ddd, J = 0.9, 7.6, 7.6 Hz, 1H, H-5), 7.25 (d, J = 7.1 Hz, 1H, H-4), 7.28 (dd, J = 7.7, 7.6 Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ 15.1 (SCH_3), 20.4 (CH_3); 64.0 (OCH_3), 70.9 (C-3), 72.8 (C-4′), 86.0 (C-2), 113.0 (C-7), 114.6 (C-2″, C-6″), 122.7 (C-4), 123.8 (C-5), 128.3 (C-4″), 129.1 (C-3a), 129.8 (C-3″,C-5″), 129.8 (C-6), 143.5 (C-1″), 148.7 (C-7a), 163.4 (C-2′). NOESY correlations: CH_3/H-3″, H-5″; OCH_3/

H-7; H-4′$_b$/H-4′$_a$, H-2; NH/H-2, H-2″, H-6″; H-4′$_a$/H-′$_b$, H-4; H-2/H-4′$_b$, NH, H-2″, H-6″; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-7/OCH$_3$, H-6; H-3″, H-5″/CH$_3$, H-2″, H-6″; H-5/H-6, H-4; H-6/H-7, H-5; H-4/H-4′$_a$, H-5. EIMS *m/z* (relative intensity) 372 M$^+$ (1%), 341 (100%), 265 (14%), 233 (22%), 161 (45%), 117 (18%), 91 (31%). Anal. Calcd for $C_{19}H_{21}N_3OS_2$ requires: C, 61.42; H, 5.70; N, 11.31. Found: C, 61.71; H, 5.83; N, 11.62.

4.1.4.3. *cis*-1-Methoxy-2-(4-methylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (13b) by isomerisation of *trans*-diastereoisomer 13a. *Procedure A*. To a stirred solution of 1-methoxybrassinin **2** (0.031 g, 0.1164 mmol) in anhydrous dichloromethane (1.3 mL) was added the solution of bromine (0.3 mL, 0.128 mmol, stock solution prepared by dissolving of 0.04 mL of Br_2 in 1.76 mL of anhydrous dichloromethane). After stirring at room temperature for 5 min a solution of 4-methylaniline (0.112 g, 1.048 mmol) and triethylamine (0.224 g, 0.31 mL, 2.212 mmol) in anhydrous dichloromethane (1.5 mL) was added. After being stirred for 10 min, the reaction mixture was diluted with dichloromethane (5 mL), successively washed with of 1 M HCl (5 mL), water (10 mL) and brine (10 mL), and the obtained dichloromethane solution was dried over Na_2SO_4. The residue obtained after the evaporation of solvent was dissolved in anhydrous dichloromethane (3.7 mL) and solution of trifluoroacetic acid (0.43 mL, 0.011 mmol, stock solution prepared by dissolving 0.01 mL TFA in 5 mL anhydrous dichloromethane) was added. After stirring for 24 h the solvent was evaporated and the residue subjected to the flash chromatography on silica gel (7 g, ethyl acetate/*n*-hexane 1:8), affording **13b** (29 mg, 67%).

Procedure B. To a stirred solution of 1-methoxybrassinin **2** (0.031 g, 0.1164 mmol) in anhydrous dichloromethane (1.3 mL) was added the solution of bromine (0.3 mL, 0.128 mmol, stock solution prepared by dissolving of 0.04 mL of Br_2 in 1.76 mL of anhydrous dichloromethane). After stirring at room temperature for 5 min a solution of *p*-toluidine (0.025 g, 0.233 mmol) in anhydrous dichloromethane (1.5 mL) was added. After 1 h the triethylamine (0.224 g, 0.31 mL, 2.212 mmol) was added and stirring was continued for another 5 min. The reaction mixture was then diluted with dichloromethane (5 mL), successively washed with 1 M HCl (5 mL), water (10 mL), and brine (10 mL). The obtained dichloromethane solution was dried over Na_2SO_4 and the residue obtained after the evaporation of solvent was subjected to the flash chromatography on silica gel (7 g, Merck 230–400 mesh), affording separated **13b** (28 mg, 65%).

4.1.4.4. *trans*- and *cis*-1-Methoxy-2-(3,4-dimethylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (14a and 14b). Following the general procedure products **14a** and **14b** were obtained using 0. 091 g (0.75 mmol) of 3,4-dimethylaniline and separated on silica gel (20 g, ethyl acetate/*n*-hexane 1:5). *trans-1-Methoxy-2-(3,4-dimethylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**14a**). Yield: 0.034 g (24%); white crystals; mp 134–135 °C (dichloromethane–hexane); R_f = 0.32 (ethyl acetate/*n*-hexane (1:5). IR ($CHCl_3$) ν_{max} 3014, 2935, 2356, 1700, 1600, 1570, 1500, 1456, 1398, 1300, 1228, 1191, 1114, 1091, 1049, 991, 735 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 2.12 (s, 3H, 4″-CH$_3$), 2.16 (s, 3H, 3″-CH$_3$), 2.48 (s, 3H, SCH$_3$), 3.78 (s, 3H, OCH$_3$), 3.88 (d, *J* = 15.6 Hz, 1H, H-4′$_a$), 4.91 (d, *J* = 15.6 Hz, 1H, H-4′$_b$), 5.31 (d, *J* = 10.2 Hz, 1H, H-2), 5.36 (d, *J* = 10.2 Hz, 1H, NH), 6.70 (dd, *J* = 2.5, 8.1 Hz, 1H, H-6″), 6.77 (d, *J* = 2.3 Hz, 1H, H-2″), 6.90 (d, *J* = 8.1 Hz, 1H, H-5″), 6.98 (ddd, *J* = 0.6, 0.9, 7.8 Hz, 1H, H-7), 7.05 (ddd, *J* = 1.1, 7.6, 7.6 Hz, 1H, H-5), 7.28 (ddd, *J* = 1.2, 7.6, 7.7 Hz, 1H, H-6), 7.34 (ddd, *J* = 0.6, 1.2, 7.6 Hz, 1H, H-4). ^{13}C NMR (acetone-d_6) δ 14.3 (SCH$_3$), 18.2 (4″-CH$_3$), 19.5 (3″-CH$_3$), 63.6 (OCH$_3$), 69.8 (C-3), 70.3 (C-4′), 90.0 (C-2), 112.1 (C-6″), 113.4 (C-7), 116.3 (C-2″), 123.7 (C-5), 124.0 (C-4), 126.0 (C-4″), 128.4 (C-3a), 129.6 (C-6), 130.2 (C-5″), 136.8 (C-3″), 145.8 (C-1″), 149.4 (C-7a), 163.0 (C-2′). NOESY correlations: 4″-CH$_3$/H-5″; 3″-CH$_3$/H-2″; OCH$_3$/H-7; H-4′$_a$/H-4′$_b$, H-4; H-4′$_b$/H-4′$_a$, NH; H-2/NH, H-6″, H-2″; NH/H-4′$_b$, H-2, H-6″, H-2″; H-6″/H-2, NH, H-2″, H-5″; H-2″/3″-CH$_3$, H-2, NH, H-6″; H-5″/4″-CH$_3$, H-6″; H-7/OCH$_3$, H-6; H-5/H-6, H-4; H-6/H-7, H-5; H-4/H-4′$_a$, H-5. EIMS *m/z* (relative intensity) 386 M$^+$ (1%), 355 (100%), 266 (8%), 233 (11%), 161 (21%), 121 (21%), 117 (24%), 77 (26%). Anal. Calcd for $C_{20}H_{23}N_3OS_2$ requires: C, 62.30; H, 6.01; N, 10.90. Found: C, 62.63; H, 6.28; N, 10.59. *cis-1-Methoxy-2-(3,4-dimethylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**14b**). Yield: 0.048 g (33%); yellow oil; R_f = 0.50 (ethyl acetate/*n*-hexane (1:5). IR ($CHCl_3$) ν_{max} 3014, 2935, 1700, 1614, 1570, 1500, 1456, 1400, 1300, 1256, 1191, 1128, 1091, 1049, 998, 735 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 2.13 (s, 3H, 4″-CH$_3$), 2.17 (s, 3H, 3″-CH$_3$), 2.51 (s, 3H, SCH$_3$), 3.72 (s, 3H, OCH$_3$), 4.30 (d, *J* = = 15.8 Hz, 1H, H-4′$_b$), 4.70 (d, *J* = 15.8 Hz, 1H, H-4′$_a$), 5.04 (d, *J* = 11.0 Hz, 1H, NH), 5.12 (d, *J* = 11.0 Hz, 1H, H-2), 6.77 (dd, *J* = 2.4, 8.1 Hz, 1H, H-6″), 6.84 (d, *J* = 2.2 Hz, 1H, H-2″), 6.92 (d, *J* = 8.1 Hz, 1H, H-5″), 6.97 (d, *J* = 8.0 Hz, 1H, H-7), 7.04 (ddd, *J* = 0.9, 7.5, 7.6 Hz, 1H, H-5), 7.29 (dd, *J* = 1.1, 7.6 Hz, 1H, H-4), 7.29 (ddd, *J* = 1.1, 7.5, 7.9 Hz, 1H, H-6). ^{13}C NMR (acetone-d_6) δ 15.0 (SCH$_3$), 18.8 (4″-CH$_3$), 20.0 (3″-CH$_3$), 64.2 (OCH$_3$), 72.1 (C-3), 73.4 (C-4′), 86.9 (C-2), 112.8 (C-6″), 113.7 (C-7), 117.1 (C-2″), 123.6 (C-4), 124.4 (C-5), 127.0 (C-4″), 130.3 (C-3a), 130.4 (C-6), 130.9 (C-5″), 137.6 (C-3″), 145.6 (C-1″), 149.8 (C-7a), 162.9 (C-2′). NOESY correlations: 4″-CH$_3$/H-5″; 3″-CH$_3$-/H-2″; OCH$_3$/H-7; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; NH/H-2, H-2″; H-2/H-4′$_b$, NH, H-6″, H-2″; H-6″/H-2, H-5″; H-2″/3″-CH$_3$, NH, H-2; H-5″/4″-CH$_3$, H-6″; H-7/H-6; H-5/H-4, H-6; H-4/H-4′$_a$, H-5; H-6/H-7, H-5. EIMS *m/z* (relative intensity) 386 M$^+$ (1%), 355 (100%), 265 (14%), 233 (15%), 117 (23%), 77 (24%). Anal. Calcd for $C_{20}H_{23}N_3OS_2$ requires: C, 62.30; H, 6.01; N, 10.90. Found: C, 62.65; H, 5.86; N, 10.67.

4.1.4.5. *trans*- and *cis*-1-Methoxy-2-(4-methoxyphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (15a and 15b). Following the general procedure products **15a** and **15b** were obtained using 0.092 g (0.75 mmol) of 4-methoxylaniline and separated on silica gel (20 g, ethyl acetate/*n*-hexane 1:5). *trans-1-Methoxy-2-(4-methoxyphenylamino)-2′-(methylsulphanyl)-spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**15a**). Yield: 0.026 g (18%); white crystals; mp 143–145 °C (acetone–hexane); R_f = 0.13 (ethyl acetate/*n*-hexane, 1:5). IR ($CHCl_3$) ν_{max} 3000, 2300, 1614, 1514, 1456, 1400, 1314, 1235, 1100, 998, 949, 814, 614 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.50 (s, 3H, SCH$_3$), 3.76 (s, 3H, 4″-OCH$_3$), 3.80 (s, 3H, N–OCH$_3$), 3.95 (d, *J* = 15.7 Hz, 1H, H-4′$_a$), 3.97 (d, *J* = 9.9 Hz, 1H, NH), 4.81 (d, *J* = 15.7 Hz, 1H, H-4′$_b$), 5.15 (d, *J* = 9.9 Hz, 1H, H-2), 6.79 (d, *J* = 9.0 Hz, 2H, H-3″, H-5″), 6.82 (d, *J* = 9.0 Hz, 2H, H-2″, H-6″), 6.98 (d, *J* = 7.8 Hz, 1H, H-7), 7.04 (dd, *J* = 7.5, 7.5 Hz, 1H, H-5), 7.27 (dd, *J* = 7.7, 7.8 Hz, 1H, H-6), 7.35 (d, *J* = 7.5 Hz, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ 15.1 (SCH$_3$), 55.7 (4″-OCH$_3$), 64.0 (N–OCH$_3$), 69.4 (C-3), 69.8 (C-4′), 91.6 (C-2), 113.4 (C-7), 114.7 (C-3″, C-5″), 116.2 (C-2″, C-6″), 123.7 (C-5), 123.8 (C-4), 127.0 (C-3a), 129.6 (C-6), 140.6 (C-1″), 149.2 (C-7a), 153.2 (C-4″), 166.1 (C-2′). NOESY correlations: 4″-OCH$_3$/H-3″, H-5″; N-OCH$_3$/H-7; H-4′$_a$/H-4′$_b$, H-4; NH/H-4′$_b$, H-2, H-2″,H-6″; H-4′$_b$/H-4′$_a$, NH; H-2/NH, H-2″, H-6″; H-3″, H-5″/4″-OCH$_3$, H-2″, H-6″; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-7/N-OCH$_3$, H-6; H-5/H-6, H-4; H-6/H-7, H-5; H-4/H-4′$_a$, H-5. EIMS *m/z* (relative intensity) 387 M$^+$ (1%), 356 (100%), 265 (28%), 233 (14%), 161 (57%), 134 (28%), 122 (22%), 117 (31%), 108 (33%). Anal. Calcd for $C_{19}H_{21}N_3O_2S_2$ requires: C, 58.89; H, 5.46; N, 10.84. Found: C, 58.61; H, 5.75; N, 10.49.

cis-1-Methoxy-2-(4-methoxyphenylamino)-2′-(methylsulphanyl)-spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (**15b**). Yield: 0.030 g (21%); white crystals; mp 178–180 °C (acetone–hexane); R_f = 0.24 (ethyl acetate/*n*-hexane, 1:5). IR ($CHCl_3$) ν_{max} 3400, 3014, 2935, 1607, 1570, 1514, 1463, 1400, 1307, 1235, 1177, 1100, 1049, 998, 949, 828, 621 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 2.52 (s, 3H, SCH$_3$), 3.72 (s,

3H, 4″-OCH_3), 3.72 (s, 3H, N–OCH_3), 4.32 (d, *J* = 15.8 Hz, 1H, H-4′$_b$), 4.71 (d, *J* = 15.8 Hz, 1H, H-4′$_a$), 5.00 (d, *J* = 11.1 Hz, 1H, NH), 5.07 (d, *J* = 11.1 Hz, 1H, H-2), 6.79 (d, *J* = 9.0 Hz, 2H, H-3″, H-5″), 6.97 (dd, *J* = 1.1, 8.1 Hz, 1H, H-7), 7.00 (d, *J* = 9.0 Hz, 2H, H-2″, H-6″), 7.05 (ddd, *J* = 1.1, 7.6, 7.8 Hz, 1H, H-5), 7.28 (dd, *J* = 1.2, 7.8 Hz, 1H, H-4), 7.29 (ddd, *J* = 1.2, 7.5, 7.8 Hz,1H, H-6). ^{13}C NMR ^{1}H NMR (acetone-d_6) δ 15.0 (SCH_3), 55.7 (4″-OCH_3), 64.2 (N–OCH_3), 72.1 (C-3), 73.5 (C-4′), 87.9 (C-2), 113.8 (C-7), 115.3 (C-2″, C-6″), 116.8 (C-3″, C-5″), 123.6 (C-4), 124.4 (C-5), 130.4 (C-6), 130.4 (C-3a), 141.4 (C-1″), 149.9 (C-7a), 153.9 (C-4″), 163.0 (C-2′). NOESY correlations: 4″-OCH_3/H-3″, H-5″; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; NH/H-2, H-2″, H-6″; H-2/H-4′$_b$, NH, H-2″, H-6″; H-3″, H-5″/4″-OCH_3, H-2″, H-6″; H-7/H-6; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-5/H-4, H-6; H-4/H-4′$_a$, H-5; H-6/H-7, H-5. EIMS *m/z* (relative intensity) 387 M^+ (1%), 356 (100%), 265 (22%), 233 (11%), 161 (62%), 134 (27%), 122 (28%), 117 (34%), 108 (31%). Anal. Calcd for $C_{19}H_{21}N_3O_2S_2$ requires: C, 58.89; H, 5.46; N, 10.84. Found: C, 59.06; H, 5.31; N, 11.07.

4.1.4.6. *trans*- and *cis*-1-Methoxy-2-(2-chlorophenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (16a and 16b). Following the general procedure products **16a** and **16b** were obtained using 0.096 g (0.08 mL, 0.75 mmol) of 2-chloroaniline and separated on silica gel (20 g, ethyl acetate/*n*-hexane 1:8). Fractions of **16a** contained small amount of 2-chloroaniline as an impurity which was removed by repeated chromatography on silica gel (5 g, dichloromethane/*n*-hexane 1:1). *trans-1-Methoxy-2-(2-chlorophenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**16a**). Yield: 0.052 g (35%); yellow oil; R_f = 0.44 (ethyl acetate/*n*-hexane (1:8). IR ($CHCl_3$) ν_{max} 3360, 3167, 1733, 1587, 1453, 1120, 1033, 940, 740 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.48 (s, 3H, SCH_3), 3.83 (s, 3H, OCH_3), 3.96 (d, *J* = 15.7 Hz, 1H, H-4′$_a$), 4.89 (d, *J* = 15.7 Hz, 1H, H-4′$_b$), 5.06 (d, *J* = 9.2 Hz, 1H, NH), 5.27 (d, *J* = 9.2 Hz, 1H, H-2), 6.70–6.74 (m, 1H, H-4″), 7.00 (dd, *J* = 0.8, 7.8 Hz, 1H, H-7), 7.07 (ddd, *J* = 1.0, 7.6, 7.6 Hz, 1H, H-5), 7.14–7.16 (m, 2H, H-5″, H-6″), 7.24–7.29 (m, 1H, H-3″), 7.29 (ddd, *J* = 1.2, 7.6, 7.8 Hz, 1H, H-6), 7.38 (dd, *J* = 1.2, 7.6 Hz, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ 15.0 (SCH_3), 64.3 (OCH_3), 69.7 (C-3), 70.0 (C-4′), 89.7 (C-2), 113.4 (C-7), 113.7 (C-6″), 119.0 (C-4″), 120.0 (C-2″), 123.9 (C-4), 124.0 (C-5), 126.8 (C-3a), 127.8 (C-5″), 129.2 (C-3″), 129.7 (C-6), 142.6 (C-1″), 149.0 (C-7a), 165.6 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_a$/H-4′$_b$, H-4; H-4′$_b$/H-4′$_a$, NH; NH/H-4′$_b$, H-2, H-6″; H-2/NH, H-6″; H-4″/H-5″, H-3″; H-7/OCH_3, H-6; H-5/H-6, H-4; H-5″/H-4″; H-6″/NH, H-2; H-3″/H-4″; H-6/H-7, H-5; H-4/H-4′$_a$, H-5. EIMS *m/z* (relative intensity) 391 M^+ (1%), 360 (100%), 265 (13%), 233 (20%), 161 (39%), 150 (30%), 138 (28%), 127 (32%), 117 (48%). Anal. Calcd for $C_{18}H_{18}ClN_3OS_2$ requires: C, 55.16; H, 4.63; N, 10.72. Found: C, 54.88; H, 4.50; N, 10.84. *cis*-1-Methoxy-2-(2-chlorophenylamino)-2'-(methylsulphanyl)spiro{indoline-3,5'-[4',5']dihydrotiazole} (**16b**). Yield: 0.036 g (25%); yellow oil; R_f = 0.26 (ethyl acetate/*n*-hexane (1:8). IR (CHCl3) ν_{max} 3407, 3173, 1580, 1507, 1453, 1387, 1113, 933 cm^{-1}. ^{1}H NMR (CDCl3) δ 2.53 (s, 3H, SCH_3), 3.74 (s, 3H, OCH_3), 4.25 (d, *J* = 15.7 Hz, 1H, H-4′$_b$), 4.71 (d, *J* = 15.7 Hz, 1H, H-4′$_a$), 4.98 (d, *J* = 10.3 Hz, 1H, H-2), 5.67 (d, *J* = 10.3 Hz, 1H, NH), 6.73 (ddd, *J* = 1.8, 6.9, 7.9 Hz, 1H, H-4″), 6.98 (d, *J* = 7.7 Hz, 1H, H-7), 7.06 (ddd, *J* = 1.1, 7.7, 7.8 Hz, 1H, H-5), 7.13 (ddd, *J* = 1.3, 6.9, 8.3 Hz, 1H, H-5″), 7.17 (dd, *J* = 1.8, 8.3 Hz, 1H, H-6″), 7.29 (dd, *J* = 1.3, 7.9 Hz, 1H, H-3″), 7.30 (dd, *J* = 1.3, 7.8 Hz, 1H, H-4), 7.30 (ddd, *J* = 1.2, 7.6, 7.8 Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ 15.2 (SCH_3), 64.1 (OCH_3), 70.7 (C-3), 73.2 (C-4′), 85.3 (C-2), 113.0 (C-7), 113.5 (C-6″), 119.1 (C-4″), 120.3 (C-2″), 122.9 (C-4), 124.0 (C-5), 127.8 (C-5″), 128.5 (C-3a), 129.4 (C-3″), 130.0 (C-6), 142.1 (C-1″), 148.8 (C-7a), 163.9 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; H-2/H-4′$_b$, NH, H-6″; NH/H-2; H-4″/H-5″, H-3″; H-7/OCH_3, H-6; H-5/H-4, H-6; H-5″/H-4″, H-6″; H-6″/H-2, H-5″; H-3″/H-4″; H-4/H-4′$_a$, H-5; H-6/H-7, H-5. EIMS *m/z* (relative intensity) 391 M^+ (1%), 360 (100%), 265 (10%), 233 (15%), 161 (26%), 150 (24%), 138 (25%), 127 (25%), 117 (45%). Anal. Calcd for $C_{18}H_{18}ClN_3OS_2$ requires: C, 55.16; H, 4.63; N, 10.72. Found: C, 55.39; H, 4.36; N, 10.98.

4.1.4.7. *trans*- and *cis*-1-Methoxy-2-(4-chlorophenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (17a and 17b). Following the general procedure products **17a** and **17b** were obtained using 0.431 g (3.375 mmol) of 4-chloroaniline and separated on silica gel (20 g, ethyl acetate/cyclohexane 1:5). *trans*-1-*Methoxy-2-(4-chlorophenylamino)-2'-(methylsulphanyl)spiro-{indoline-3,5'-[4',5']dihydrotiazole}* (**17a**). Yield: 0.024 g (16%); white crystals; mp 162–164 °C (dichloromethane–hexane); R_f = 0.39 (ethyl acetate/cyclohexane, 1:5). IR ($CHCl_3$) ν_{max} 3400, 3000, 2362, 1600, 1563, 1491, 1456, 1400, 1284, 1177, 1098, 1050, 998, 942, 805, 620 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.49 (s, 3H, SCH_3), 3.80 (s, 3H, OCH_3), 3.94 (d, *J* = 15.7 Hz, 1H, H-4′$_a$), 4.22 (d, *J* = 9.9 Hz, 1H, NH), 4.78 (d, *J* = 15.7 Hz, 1H, H-4′$_b$), 5.19 (d, *J* = 9.9 Hz, 1H, H-2), 6.79 (d, *J* = 8.8 Hz, 2H, H-2″, H-6″), 6.99 (d, *J* = 7.8 Hz, 1H, H-7), 7.06 (ddd, *J* = 1.0, 7.5, 7.6 Hz, 1H, H-5), 7.15 (d, *J* = 8.8 Hz, 2H, H-3″, H-5″), 7.29 (ddd, *J* = 1.2, 7.5, 7.8 Hz, 1H, H-6), 7.36 (dd, *J* = 1.2, 7.6 Hz, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ 15.1 (SCH_3), 64.0 (OCH_3), 69.3 (C-3), 69.7 (C-4′), 90.5 (C-2), 113.4 (C-7), 115.7 (C-2″, C-6″), 123.8 (C-4), 123.9 (C-5), 123.9 (C-4″), 126.5 (C-3a), 129.1 (C-3″, C-5″), 129.8 (C-6), 145.2 (C-1″), 149.1 (C-7a), 166.4 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_a$/H-4′$_b$, H-4; NH/H-4′$_b$, H-2, H-2″, H-6″; H-4′$_b$/H-4′$_a$, NH; H-2/NH, H-2″, H-6″; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-7/H-6; H-5/H-6, H-4; H-3″, H-5″/H-2″, H-6″; H-6/H-7, H-5; H-4/H-$_a$, H-5. MALDI-TOF MS: 430.1 $[M+K]^+$ (6%), 413.7 $[M+Na]^+$ (10%), 392.8 $[M+H]^+$ (4%), 262.0 (10%), 283.9 (19%), 266.2 (13%), 200.3 (36%), 156.6 (19%), 138.6 (30%). Anal. Calcd for $C_{18}H_{18}ClN_3OS_2$ requires: C, 55.16; H, 4.63; N, 10.72. Found: C, 55.19; H, 4.41; N, 10.60. *cis-1-methoxy-2-(4-chlorophenylamino)-2'-(methylsulphanyl)spiro{indoline-3,5'-[4',5']dihydrotiazole}* (**17b**). Yield: 0.042 g (29%); bright yellow oil; R_f = 0.39 (ethyl acetate/cyclohexane, 1:5). IR ($CHCl_3$) ν_{max} 3400, 3000, 2935, 2300, 1598, 1498, 1456, 1400, 1300, 1235, 1177, 1091, 1042, 982, 942, 814, 614 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.54 (s, 3H, SCH_3), 3.73 (s, 3H, OCH_3), 4.22 (d, *J* = 15.7 Hz, 1H, H-4′$_b$), 4.70 (d, *J* = 15.7 Hz, 1H, H-4′$_a$), 4.81 (d, *J* = 10.7 Hz, 1H, NH), 4.92 (d, *J* = 10.7 Hz, 1H, H-2), 6.86 (d, *J* = 8.8 Hz, 2H, H-2″, H-6″), 6.97 (d, *J* = 7.8 Hz, 1H, H-7), 7.05 (ddd, *J* = 0.8, 7.5, 7.6 Hz, 1H, H-5), 7.16 (d, *J* = 8.8 Hz, 2H, H-3″,H-5″), 7.27 (d, *J* = 7.7 Hz, 1H, H-4), 7.30 (dd, *J* = 7.7, 7.7 Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ 15.1 (SCH_3), 64.1 (OCH_3), 70.7 (C-3), 73.0 (C-4′), 85.5 (C-2), 113.1 (C-7), 115.6 (C-2″, C-6″), 122.8 (C-4), 123.7 (C-4″), 124.0 (C-5), 128.7 (C-3a), 129.2 (C-3″, C-5″), 130.0 (C-6), 144.5 (C-1″), 148.6 (C-7a), 163.4 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; NH/H-2, H-2″, H-6″; H-2/NH, H-2″, H-6″; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-7/OCH_3, H-6; H-5/H-4, H-6; H-3″, H-5″/H-2″, H-6″; H-4/H-4′$_a$, H-5; H-6/H-7, H-5. MALDI-TOF MS: 430.1 $[M+K]^+$ (8%), 413.5 $[M+Na]^+$ (12%), 392 $[M+H]^+$ (13%), 361.8 (20%), 285.7 (25%), 265.9 (36%), 200.3 (39%), 161.5 (20%), 138.6 (15%). Anal. Calcd for $C_{18}H_{18}ClN_3OS_2$ requires: C, 55.16; H, 4.63; N, 10.72. Found: C, 54.87; H, 4.90; N, 10.56.

4.1.4.8. *trans*- and *cis*-1-methoxy-2-(3,4-dichlorophenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (18a and 18b). Following the general procedure products **18a** and **18b** were obtained using 0.122 g (0.75 mmol) of 3,4-dichloroaniline and separated on silica gel (20 g, ethyl acetate/*n*-hexane 1:8). Fractions of **18a** contained small amount of 3,4-dichloroaniline as an impurity which was removed by repeated chromatography on silica gel (5 g, dichloromethane).

trans-1-Methoxy-2-(3,4-dichlorophenylamino)-2'-(methylsulphanyl)-spiro{indoline-3,5'-[4',5']dihydrotiazole} (***18a***). Yield: 0.045 g (28%); white crystals; mp 156–158 °C (dichloromethane–hexane); R_f = 0.3 (ethyl acetate/*n*-hexane, 1:8). IR ($CHCl_3$) ν_{max} 3400, 3014,

2935, 1700, 1591, 1556, 1456, 1398, 1356, 1300, 1121, 1091, 991, 942 cm^{-1}. ^1H NMR (acetone-d_6) δ 2.47 (s, 3H, SCH_3), 3.82 (s, 3H, OCH_3), 3.89 (d, J = 15.6 Hz, 1H, H-4′$_a$), 4.87 (d, J = 15.6 Hz, 1H, H-4′$_b$), 5.41 (d, J = 10.3 Hz, 1H, H-2), 6.13 (d, J = 10.3 Hz, 1H, NH), 6.98 (dd, J = 2.7, 8.8 Hz, 1H, H-6″), 7.01 (d, J = 7.8 Hz, 1H, H-7), 7.07 (ddd, J = 1.0, 7.5, 7.5 Hz, 1H, H-5), 7.20 (d, J = 2.7 Hz, 1H, H-2″), 7.30 (d, J = 8.6 Hz, 1H, H-5″), 7.30 (ddd, J = 1.2, 7.6, 7.5 Hz, 1H, H-6), 7.36 (d, J = 7.6 Hz, 1H, H-4). ^{13}C NMR (acetone-d_6) δ 15.0 (SCH_3), 64.4 (OCH_3), 70.5 (C-3), 70.7 (C-4′), 89.5 (C-2), 114.1 (C-7), 115.1 (C-6″), 116.3 (C-2″), 120.6 (C-4″), 124.6 (C-5), 124.6 (C-4), 128.4 (C-3a), 130.4 (C-6), 131.4 (C-5″), 132.8 (C-3″), 148.6 (C-1″), 149.9 (C-7a), 163.9 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_a$/H-4′$_b$, H-4; H-4′$_b$/H-4′$_a$, NH; H-2/NH, H-2″, H-6″; NH/H-4′$_b$, H-2, H-2″, H-6″; H-6″/H-2, NH, H-5″; H-5/H-6, H-4; H-4/H-4′$_a$, H-5. EIMS m/z (relative intensity) 426 M^+ (1%), 396 (46%), 394 (61%), 265 (25%), 233 (28%), 163 (25%), 161 (100%), 150 (29%), 145 (21%), 117 (52%), 90 (20%), 72 (32%). Anal. Calcd for $C_{18}H_{17}Cl_2N_3OS_2$ requires: C, 50.70; H, 4.02; N, 9.86. Found: C, 51.02; H, 3.79; N, 9.53. *cis-1-Methoxy-2-(3,4-dichlorophenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**18b**). Yield: 0.049 g (31%); yellow oil; R_f = 0.36 (ethyl acetate/*n*-hexane, 1:8). IR ($CHCl_3$) ν_{max} 3400, 3000, 2827, 1733, 1593, 1493, 1387, 1127, 987, 580 cm^{-1}. ^1H NMR (acetone-d_6) δ 2.51 (s, 3H, SCH_3), 3.75 (s, 3H, OCH_3), 4.39 (d, J = 15.9 Hz, 1H, H-4′$_b$), 4.71 (d, J = 15.9 Hz, 1H, H-4′$_a$), 5.24 (d, J = 10.9 Hz, 1H, H-2), 5.81 (d, J = 10.9 Hz, 1H, NH), 7.00 (dd, J = 0.9, 7.8 Hz, 1H, H-7), 7.07 (ddd, J = 1.0, 7.6, 7.7 Hz, 1H, H-5), 7.08 (dd, J = 2.7, 8.8 Hz, 1H, H-6″), 7.31 (ddd, J = 1.2, 7.6, 7.6 Hz, 1H, H-6), 7.31 (d, J = 8.8 Hz, 1H, H-5″), 7.33 (d, J = 2.7 Hz, 1H, H-2″), 7.33 (dd, J = 1.0, 7.6 Hz, 1H, H-4). ^{13}C NMR δ (ppm): 15.0 (SCH_3), 64.3 (OCH_3), 71.8 (C-3), 73.4 (C-4′), 85.9 (C-2), 113.8 (C-7), 115.4 (C-6″), 116.7 (C-2″), 120.9 (C-4″), 123.8 (C-4), 124.7 (C-5), 129.9 (C-3a), 130.6 (C-6), 131.4 (C-5″), 132.9 (C-3″), 148.1 (C-1″), 149.8 (C-7a), 163.1 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; H-2/H-4′$_b$, NH, H-6″, H-2″; NH/H-2, H-6″, H-2″; H-7/OCH_3, H-6; H-5/H-6, H-4; H-6″/H-2, NH, H-5″, H-2″; H-6/H-7, H-5; H-5″/H-6″; H-2″/H-2, NH, H-6″; H-4/H-4′$_a$, H-5. EIMS m/z (relative intensity) 426 M^+ (1%), 396 (68%), 394 (100%), 265 (27%), 233 (23%), 161 (85%), 150 (31%), 145 (29%), 117 (60%), 91 (36%), 72 (41%). Anal. Calcd for $C_{18}H_{17}Cl_2N_3OS_2$ requires: C, 50.70; H, 4.02; N, 9.86. Found: C, 50.49; H, 4.35; N, 10.17.

4.1.4.9. *trans*- and *cis*-1-Methoxy-2-(4-bromophenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (19a and 19b). Following the general procedure products **19a** and **19b** were obtained using 0.581 g (3.375 mmol) of 4-bromoaniline and separated on silica gel (20 g, ethyl acetate/cyclohexane 1:8). *trans-1-Methoxy-2-(4-bromophenylamino)-2′-(methylsulphanyl) spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**19a**). Yield: 0.035 g (21%); white crystals; mp 177–179 °C (acetone–hexane); R_f = 0.15 (ethyl acetate/cyclohexane, 1:8). IR ($CHCl_3$) ν_{max} 3410, 3010, 1610, 1480, 1450, 1390, 1090, 985, 935, 805, 610 cm^{-1}. ^1H NMR (Acetone-d_6) δ 2.48 (s, 3H, SCH_3), 3.80 (s, 3H, OCH_3), 3.90 (d, J = 15.7 Hz, 1H, H-4′$_a$), 4.89 (d, J = 15.7 Hz, 1H, H-4′$_b$), 5.36 (d, J = 10.3 Hz, 1H, H-2), 5.81 (d, J = 10.3 Hz, 1H, NH), 6.95 (d, J = 8.9 Hz, 2H, H-2″, H-6″), 7.00 (dd, J = 0.9, 7.8 Hz, 1H, H-7), 7.06 (ddd, J = 1.1, 7.4, 7.4 Hz, 1H, H-5), 7.28 (d, J = 8.9 Hz, 2H, H-3″, H-5″), 7.29 (ddd, J = 1.1, 7.6, 7.7 Hz, 1H, H-6), 7.35 (dd, J = 1.1, 7.4 Hz, 1H, H-4). ^{13}C NMR (acetone-d_6) δ 15.0 (SCH_3), 64.3 (OCH_3), 70.6 (C-3), 70.9 (C-4′), 90.0 (C-2), 110.2 (C-4″), 114.2 (C-7), 117.1 (C-2″, C-6″), 124.6 (C-5), 124.6 (C-4), 128.8 (C-3a), 130.4 (C-6), 132.5 (C-3″, C-5″), 147.9 (C-1″), 150.1 (C-7a), 164.0 (C-2′). NOESY correlations: H-4′$_a$/H-4′$_b$; H-4′$_b$/H-4′$_a$, NH; H-2/NH, H-2″, H-6″; NH/H-4′$_b$, H-2, H-2″, H-6″; H-2″, H-6″/H-2, NH, H-3″, H-5″; H-7/H-6; H-5/H-4, H-6; H-3″, H-5″/H-2″, H-6″; H-6/ H-7, H-5; H-4/H-5. EIMS m/z (relative intensity) 435 M^+ (1%), 436 M^+ (1%), 406 (100%), 404 (94%), 265 (41%), 233 (35%), 218 (26%), 161 (68%), 150 (44%), 117 (62%). Anal. Calcd for $C_{18}H_{18}BrN_3OS_2$ requires: C, 49.54; H, 4.16; N, 9.63. Found: C, 49.24; H, 4.35; N, 9.32.

cis-1-Methoxy-2-(4-bromophenylamino)-2′-(methylsulphanyl)-spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (**19b**). Yield: 0.070 g (43%); bright yellow oil; R_f = 0.35 (ethyl acetate/cyclohexane, 1:8). IR ($CHCl_3$) ν_{max} 3380, 2973, 2933, 1573, 1486, 1400, 1300, 1226, 947, 930, 800, 733 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.53 (s, 3H, SCH_3), 3.73 (s, 3H, OCH_3), 4.22 (d, J = 15.7 Hz, 1H, H-4′$_b$), 4.69 (d, J = 15.7 Hz, 1H, H-4′$_a$), 4.82 (d, J = 10.7 Hz, 1H, NH), 4.92 (d, J = 10.7 Hz, 1H, H-2), 6.81 (d, J = 8.9 Hz, 2H, H-2″, H-6″), 6.97 (d, J = 7.8 Hz, 1H, H-7), 7.05 (ddd, J = 1.1, 7.6, 7.6 Hz, 1H, H-5), 7.26 (d, J = 7.6 Hz, 1H, H-4), 7.29 (d, J = 8.9 Hz, 2H, H-3″, H-5″), 7.29 (ddd, J = 1.2, 7.5, 7.8 Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ 15.4 (SCH_3), 64.3 (OCH_3), 71.0 (C-3), 73.3 (C-4′), 85.7 (C-2), 111.1 (C-4″), 113.3 (C-7), 116.4 (C-2″, C-6″), 123.1 (C-4), 124.3 (C-5), 129.0 (C-3a), 130.2 (C-6), 132.3 (C-3″,C-5″), 145.2 (C-1″), 148.9 (C-7a), 163.6 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; NH/H-2, H-2″, H-6″; H-2/H-4′$_b$, NH, H-2″, H-6″; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-7/OCH_3, H-6; H-5/H-4, H-6; H-4/H-4′$_a$, H-5; H-3″, H-5″/H-2″, H-6″; H-6/H-7, H-5. EIMS m/z (relative intensity) 435 M^+ (1%), 436 M^+ (1%), 406 (100%), 404 (87%), 265 (28%), 233 (20%), 161 (38%), 150 (28%), 117 (51%), 91 (25%), 76 (22%). Anal. Calcd for $C_{18}H_{18}BrN_3OS_2$ requires: C, 49.54; H, 4.16; N, 9.63. Found: C, 49.29; H, 4.41; N, 9.43.

4.1.4.10. *trans*- and *cis*-1-Methoxy-2-(4-nitrophenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (20a and 20b). Following the general procedure products **20a** and **20b** were obtained using 0.077 g (0.563 mmol) of 4-nitroaniline and separated on silica gel (20 g, ethyl acetate/*n*-hexane 1:2). Fractions of **20a** contained small amount of 4-nitroaniline as an impurity which was removed by repeated chromatography on silica gel (5 g, dichloromethane). *trans-1-Methoxy-2-(4-nitrophenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**20a**). Yield: 0.020 g (13%); yellow crystals; mp 184–186 °C (ethyl acetate-hexane); R_f = 0.40 (ethyl acetate/*n*-hexane, 1:2). IR ($CHCl_3$) ν_{max} 3400, 3133, 3013, 1700, 1587, 1313, 1160, 1107, 933, 827 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.47 (s, 3H, SCH_3), 3.82 (s, 3H, OCH_3), 3.95 (d, J = 15.8 Hz, 1H, H-4′$_a$), 4.77 (d, J = 15.8 Hz, 1H, H-4′$_b$), 5.03 (d, J = 9.9 Hz, 1H, NH), 5.35 (d, J = 9.9 Hz, 1H, H-2), 6.88 (d, J = 9.2 Hz, 2H, H-2″, H-6″), 7.02 (d, J = 7.9 Hz, 1H, H-7), 7.10 (ddd, J = 1.0, 7.5, 7.6 Hz, 1H, H-5), 7.32 (ddd, J = 1.2, 7.6, 7.8 Hz, 1H, H-6), 7.39 (d, J = 7.6 Hz, 1H, H-4), 8.12 (d, J = 9.2 Hz, 2H, H-3″, H-5″). ^{13}C NMR ($CDCl_3$) δ 15.3 (SCH_3), 64.5 (OCH_3), 69.8 (C-4′), 69.9 (C-3), 89.1 (C-2), 113.2 (C-2″, C-6″), 113.9 (C-7), 124.1 (C-4), 124.6 (C-5), 126.1 (C-3a), 126.4 (C-3″, C-5″), 130.3 (C-6), 139.9 (C-4″), 149.2 (C-7a), 152.4 (C-1″), 166.9 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_a$/H-4′$_b$, H-4; H-4′$_b$/H-4′$_a$, NH; NH/H-4′$_b$, H-2, H-2″, H-6″; H-2/NH, H-2″, H-6″; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-7/OCH_3; H-5/H-6, H-4; H-6/H-5; H-4/H-$_a$, H-5; H-3″, H-5″/H-2″, H-6″. EIMS m/z (relative intensity) 403 M^+ (1%), 371 (100%), 323 (14%), 265 (13%), 233 (13%), 160 (26%), 149 (22%), 117 (30%). Anal. Calcd for $C_{18}H_{18}N_4O_3S_2$ requires: C, 53.71; H, 4.51; N, 13.92. Found: C, 53.99; H, 4.35; N, 13.74.

cis-1-Methoxy-2-(4-nitrophenylamino)-2′-(methylsulphanyl)-spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (**20b**). Yield: 0.035 g (23%); bright yellow oil; R_f = 0.63 (ethyl acetate/*n*-hexane, 1:2). IR ($CHCl_3$) ν_{max} 3391, 3014, 2363, 1714, 1598, 1500, 1400, 1321, 1249, 1184, 1114, 1049, 991, 942, 742 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.54 (s, 3H, SCH_3), 3.74 (s, 3H, OCH_3), 4.25 (d, J = 15.8 Hz, 1H, H-4′$_b$), 4.73 (d, J = 15.8 Hz, 1H, H-4′$_a$), 5.04 (d, J = 10.5 Hz, 1H, H-2), 5.52 (d, J = 10.5 Hz, 1H, NH), 6.95 (d, J = 9.2 Hz, 2H, H-2″, H-6″), 7.00 (d, J = 7.9 Hz, 1H, H-7), 7.10 (ddd, J = 0.7, 7.6, 7.6 Hz, 1H, H-5), 7.30 (d, J = 7.9 Hz, 1H, H-4), 7.34 (ddd, J = 0.9, 7.6, 7.8 Hz, 1H, H-6), 8.14 (d, J = 9.2 Hz, 2H, H-3″, H-5″). ^{13}C NMR ($CDCl_3$) δ 15.2

(SCH_3), 64.2 (OCH_3), 70.7 (C-3), 73.1 (C-4′), 83.9 (C-2), 113.2 (C-7), 113.2 (C-2″, C-6″), 123.0 (C-4), 124.4 (C-5), 126.2 (C-3″, C-5″), 128.0 (C-3a), 130.3 (C-6), 139.8 (C-4″), 148.5 (C-7a), 151.5 (C-1″), 163.4 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; H-2/H-4′$_b$, NH, H-2″, H-6″; NH/H-2, H-2″, H-6″; H-2″, H-6″/H-2, NH, H-3″, H-5″; H-7/OCH_3, H-6; H-5/H-6, H-4; H-6/H-7, H-5; H-4/H-5, H-3″, H-5″/H-2″, H-6″. EIMS *m/z* (relative intensity) 403 M^+ (1%), 371 (100%), 265 (19%), 233 (16%), 161 (35%), 160 (30%), 149 (56%), 117 (56%). Anal. Calcd for $C_{18}H_{18}N_4O_3S_2$ requires: C, 53.71; H, 4.51; N, 13.92. Found: C, 53.95; H, 4.83; N, 14.12.

4.1.4.11. *trans*- and *cis*-1-Methoxy-2-(4-trifluoromethylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole} (21a and 21b). Following the general procedure products **21a** and **21b** were obtained using 0.121 g (0.094 mL, 0.75 mmol) of 4-trifluoromethylaniline and separated on silica gel (20 g, ethyl acetate/*n*-hexane 1:5). Fractions of **21a** contained small amount of 4-trifluoromethylaniline as an impurity which was removed by repeated chromatography on silica gel (5 g, dichloromethane/*n*-hexane 1:1). *trans-1-Methoxy-2-(4-trifluoromethylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]-dihydrotiazole}* (**21a**). Yield: 0.037 g (23%); white crystals; mp 114–116 °C (acetone–hexane); R_f = 0.13 (ethyl acetate/*n*-hexane (1:5). IR ($CHCl_3$) ν_{max} 3414, 2970, 1700, 1607, 1556, 1528, 1456, 1400, 1314, 1256, 1163, 1114, 1070, 991, 942, 735 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.47 (s, 3H, SCH_3), 3.80 (s, 3H, OCH_3), 3.95 (d, *J* = 15.8 Hz,1H, H-4′$_a$), 4.53 (d, *J* = 9.9 Hz, 1H, NH), 4.78 (d, *J* = 15.8 Hz, 1H, H-4′$_b$), 5.30 (d, *J* = 9.9 Hz, 1H, H-2), 6.90 (d, *J* = 8.5 Hz, 2H, H-2″, H-6″), 7.00 (d, *J* = 7.8 Hz, 1H, H-7), 7.07 (ddd, *J* = 1.0, 7.5, 7.6 Hz, 1H, H-5), 7.30 (ddd, *J* = 1.1, 7.6, 7.8 Hz, 1H, H-6), 7.38 (d, *J* = 7.6 Hz, 1H, H-4), 7.44 (d, *J* = 8.5 Hz, 2H, H-3″, H-5″). ^{13}C NMR ($CDCl_3$) δ 15.3 (SCH_3), 64.4 (OCH_3), 69.7 (C-3), 69.9 (C-4′), 89.8 (C-2), 113.8 (C-7), 113.9 (C-2″, C-6″), 121.1 (q, *J* = 32.7 Hz, C-4″), 124.1 (C-4), 124.3 (C-5), 124.9 (q, *J* = 270.6 Hz, CF_3), 126.5 (C-3a), 126.9 (C-3″, C-5″), 130.1 (C-6), 149.3 (C-7a), 149.5 (C-1″), 166.9 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_a$/H-4′$_b$, H-4; NH/H-4′$_b$, H-2, H-2″, H-6″; H-4′$_b$/H-4′$_a$, NH; H-2/NH, H-2″,H-6″; H-2″, H-6″/NH, H-2, H-3″, H-5″; H-7/OCH_3, H-6; H-5/H-6, H-4; H-6/H-7, H-5; H-4/H-4′$_a$, H-5; H-3″; H-5″/H-2″, H-6″. EIMS *m/z* (relative intensity) 426 M^+ (1%), 395 (100%), 265 (11%), 233 (11%), 161 (25%), 150 (22%), 117 (29%). Anal. Calcd for $C_{19}H_{18}F_3N_3OS_2$ requires: C, 53.63; H, 4.26; N, 9.88. Found: C, 53.91; H, 4.63; N, 9.57. *cis-1-Methoxy-2-(4-trifluoromethylphenylamino)-2′-(methylsulphanyl)spiro{indoline-3,5′-[4′,5′]dihydrotiazole}* (**21b**). Yield: 0.070 g (44%); yellow oil; R_f = 0.28 (ethyl acetate/*n*-hexane (1:5). IR ($CHCl_3$) ν_{max} 3391, 2984, 2928, 1700, 1614, 1570, 1528, 1500, 1456, 1400, 1314, 1256, 1191, 1163, 1114, 1070, 991, 942, 828, 735 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.53 (s, 3H, SCH_3), 3.74 (s, 3H, OCH_3), 4.23 (d, *J* = 15.7 Hz, 1H, H-4′$_b$), 4.71 (d, *J* = 15.7 Hz, 1H, H-4′$_a$), 5.01 (d, *J* = 10.6 Hz, 1H, H-2), 5.16 (d, *J* = 10.6 Hz, 1H, NH), 6.97 (d, *J* = 8.4 Hz, 2H, H-2″, H-6″), 6.98 (d, *J* = 7.7 Hz, 1H, H-7), 7.07 (ddd, *J* = 1.1, 7.6, 7.6 Hz, 1H, H-5), 7.28 (d, *J* = 7.2 Hz, 1H, H-4), 7.31 (ddd, *J* = 1.2, 7.6, 7.7 Hz, 1H, H-6), 7.45 (d, *J* = 8.4 Hz, 2H, H-3″, H-5″). ^{13}C NMR ($CDCl_3$) δ 15.1 (SCH_3), 64.1 (OCH_3), 70.8 (C-3), 73.1 (C-4′), 84.6 (C-2), 113.2 (C-7), 113.7 (C-2″, C-6″), 121.1 (q, *J* = 32.7 Hz, C-4″), 122.9 (C-4), 124.1 (C-5), 124.7 (q, *J* = 270.5 Hz, CF_3), 126.7 (C-3″, C-5″), 128.5 (C-3a), 130.1 (C-6), 148.6 (C-1″), 148.6 (C-7a), 163.4 (C-2′). NOESY correlations: OCH_3/H-7; H-4′$_b$/H-4′$_a$, H-2; H-4′$_a$/H-4′$_b$, H-4; H-2/NH, H-4′$_b$, H-2″, H-6″; NH/H-2, H-2″, H-6″; H-2″, H-6″/H-3″, H-5″; H-7/OCH_3, H-6; H-5/H-4, H-6; H-6/H-5, H-7; H-4/H-4′$_a$, H-5. EIMS *m/z* (relative intensity) 426 M^+ (2%), 395 (100%), 265 (10%), 233 (9%), 161 (29%), 150 (31%), 145 (28%), 117 (32%). Anal. Calcd for $C_{19}H_{18}F_3N_3OS_2$ requires: C, 53.63; H, 4.26; N, 9.88. Found: C, 53.89; H, 4.02; N, 9.63.

4.2. Molecular modeling computational protocols

The 2D molecular structures drawn and saved in MDL mol format were imported into Accelrys Discovery Studio[30] in order to convert them into 3D form and add hydrogens. The 'clean geometry' option of the program was used to remove the unwanted short contacts. The 3D structures thus obtained were saved in MDL mol format, consecutively. The geometries obtained in this way were imported into MAESTRO (Schrodinger, LLC) and submitted from there for ab initio minimization runs using the JAGUAR program[20] on DFT level with 6–31** or cc-pVTZ-pp (molecules with Br) basis sets. The geometries of the *trans*- and *cis*-diastereoisomers were optimized. Different ring conformers were generated taking into account the nonplanar geometry of the five-membered rings. Conformational alterations resulting from the sp^3 character of nitrogen also were considered, resulting thus into multiple conformations for the particular molecules under study. A comprehensive set of molecular parameters (in addition to the standard Lipinski's parameters) was obtained from QIKPROP[31] (Schrodinger, LLC) calculations. The geometries generated in the above mentioned way were used for pharmacophore modeling using the PHASE[27] (Schrodinger LLD) program. The experimental IC_{50} were used in this part of the molecular modeling allowing the evaluation of generated pharmacophores and consequent 3D QSAR models.

4.3. Anticancer activity

4.3.1. Tumor cell lines

Jurkat (human T-cell acute lymphoblastic leukemia), HeLa (human cervical adenocarcinoma) MCF-7 (human breast adenocarcinoma, estrogen receptor-positive), MDA-MB-231 (human breast adenocarcinoma, estrogen receptor-negative) and A-549 cell lines (human lung adenocarcinoma) were kindly provided by Dr. M. Hajdúch (Olomouc, Czech Republic). CCRF-CEM cell line (human T-cell acute lymphoblastic leukemia) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were routinely maintained in RPMI 1640 medium with L-Glutamine and HEPES (Jurkat, HeLa and CCR-CEM) or Dulbecco's modified Eagle's medium with Glutamax-I (MCF-7, MDA-MB-231 and A-549) supplemented with 10% fetal calf serum, penicillin (100 IU × mL^{-1}) and streptomycin (100 μg × mL^{-1}) (all from Invitrogen, USA), in humidified air with 5% CO_2 at 37 °C. Before each cytotoxicity assay, cell viability was determined by the trypan blue exclusion method and found to be greater than 95%.

4.3.2. Cytotoxicity assay

The cytostatic/cytotoxic effects of compounds was studied using the colorimetric microculture assay with the MTT endpoint.[32] Briefly, 3×10^3 (A-549, MCF-7, MDA-MB-231), 5×10^3 (HeLa) or 1×10^4 (Jurkat and CEM) cells were plated per well in 96-well polystyrene microplates (Sarstedt, Germany) in the culture medium containing tested chemicals at final concentrations of 1×10^{-4}, 5×10^{-5}, 1×10^{-5} and 1×10^{-6} mol × L^{-1}. After 72 h of incubation, 10 μL of MTT (5 mg × mL^{-1}) (Sigma, Germany) were added in each well. After an additional 4 h, during which insoluble formazan was produced, 100 μL of 10% sodium dodecylsulphate were added in each well and another 12 h were allowed for the dissolution of formazan. Absorbance was measured at 540 nm using the automated MRX microplate reader (Dynatech Laboratories, UK). The blank-corrected absorbance of the control wells was taken as 100% and the results were expressed as a percentage of the control.

4.4. Data processing and analysis

IC_{50} values (concentrations of tested agents that inhibited growth of cell cultures to 50% of the untreated control) were determined by GraphPad Prism for Windows version 5.01 (GraphPad Software, Inc.). Values of IC_{50} were not determined for a given compound and cell line if the growth inhibition was less than 50% at the highest tested concentration 100 $\mu mol \times L^{-1}$. In such cases, respective IC_{50} values were recorded in Table 2 as 100 $\mu mol \times L^{-1}$.

IC_{50} values were analyzed using an approach similar to that employed with the COMPARE algorithm.[24] Specifically, growth inhibitory profiles were created for each compound using their ($-\log IC_{50}$) values for specific cell lines after subtraction of their mean ($-\log IC_{50}$) values across all cell lines. Similarities of their profiles with profiles of the conventional anticancer agents doxorubicin, etoposide and cisplatin were subsequently determined by Spotfire DecisionSite 9.1 (TIBCO Software, Palo Alto, CA, USA), and their strength was expressed as a correlation coefficient. Classification of tested compounds according to their activity patterns against cancer cell lines was accomplished by hierarchical clustering similar to previously reported procedure.[26] Clustering algorithm was applied to ($-\log IC_{50}$) values for each compound after subtraction of mean ($-\log IC_{50}$) for that compound across all tested cell lines using Spotfire DecisionSite 9.1. The following clustering parameters were applied: WPGMA method (*Weighted* Pair-Group Method using Arithmetic averages), correlation (similarity measure), and average value (ordering function).

4.5. Statistical analysis

Statistical significance of correlation coefficients was tested with a two-tailed *t*-test and a correlation was considered significant if $p < 0.05$ (for profiles based on 6 cell lines $r > 0.81$).

Statistical significance of differences in mean IC_{50} values among cell lines for the tested compounds was determined by a two-way ANOVA and Friedman's tests followed by Dunn's multiple comparison post test. Differences were considered significant if $p < 0.05$.

In order to determine the effect of geometric (*cis*:*trans*) isomerism of the tested compounds on their anticancer potency, data were organized in contingency tables (one table per each cell line) where compounds were pooled into groups of *cis*- and *trans*-isomers and classified active ($IC_{50} < 90$ $\mu mol \times L^{-1}$) or inactive ($IC_{50} > 90$ $\mu mol \times L^{-1}$). Significance of association between *cis*:*trans* isomerism and active/inactive status was examined by Fisher's exact test and considered significant if the two-tailed *p*-value <0.05.

4.6. Antimicrobial activity

Bacterial strains used in the present study were obtained from the Czech Collection of Microorganisms (CCM, Czech Republic) and involved *Pseudomonas aeruginosa* (CCM 162/78, ATCC 27853), *Enterococcus faecalis* (CCM 4224), *Escherichia coli* (CCM 326/71, ATCC 10418), *Staphylococcus aureus* (CCM 4223, ATCC 29213), *S. pyogenes* (CCM 4425) and *B. subtilis* (CCM 4062). *Candida albicans* (ATCC 60193) was obtained from the American Type Culture Collection (ATCC, USA).

For the antimicrobial activity assay, disk diffusion[33–35] was applied with minor modifications. Overnight culture of microorganisms on blood agar was used in order to prepare the inoculum (1.5×10^8 CFU/ml; 0.5 McFarland). The experiments were carried out in Petri dishes of 80 mm diameter. Inoculum was prepared in saline and applied to the Mueller–Hinton agar or Sabouraud agar with a swab to ensure homogenous distribution of microorganisms. After 3 min, sterile paper disks (Whatman No. 3, diameter 6 mm) were layered onto the inoculated Petri dishes and soaked with tested substances or antibiotics dissolved in DMSO (20 μg/2.5 μl/disk). Microorganisms were cultured with tested substances for 24 h at 37 °C. The antimicrobial activity was evaluated as a diameter of inhibition zone around the disk with tested compound. All experiments involved clinically used antimicrobial substances (amphotericin purchased from Sigma, USA and cefotaxime from Lek, a.s. Ľubľana, Slovenia) and solvent (DMSO) as control.

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