Sesquiterpene Lactones from *Centaurea spinosa* and Their Antibacterial and Cytotoxic Activities

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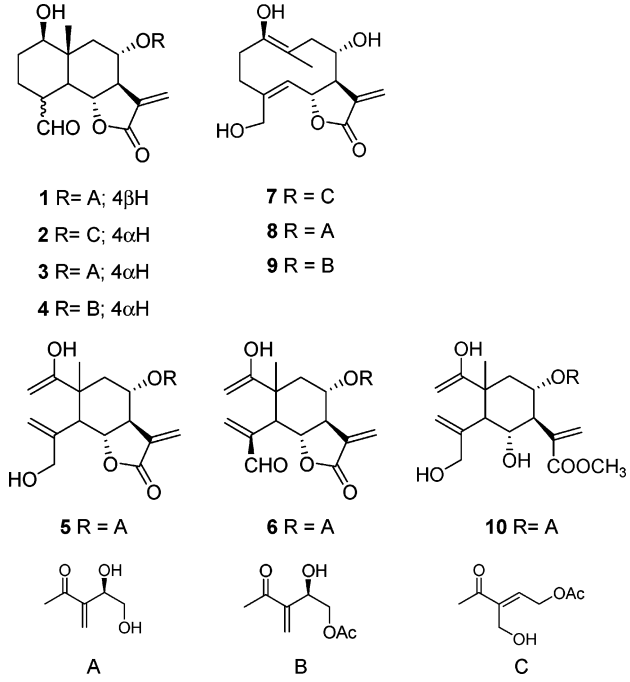
Ten sesquiterpene lactones, 8α-*O*-(3,4-dihydroxy-2-methylenebutanoyloxy)sonchucarpolide (4-*epi*-malacitanolide) (**1**), 8α-*O*-(4-acetoxy-2-hydroxymethylbuten-2-oyloxy)-4-*epi*-sonchucarpolide (**2**), malacitanolide (**3**), its 4′-acetyl derivative (**4**), 8α-*O*-(3,4-dihydroxy-2-methylenebutanoyloxy)dehydromelitensine (**5**), 8α-*O*-(3,4-dihydroxy-2-methylenebutanoyloxy)-15-oxo-5,7αH,6βH-eleman-1,3,11(13)-trien-6,12-olide (**6**), the germacranolides 8α-*O*-(4-acetoxy-2-hydroxymethylbuten-2-oyloxy)salonitenolide (**7**), cnicin (**8**), and 4′-acetylcnicin (**9**), and the sesquiterpene methyl 8α-*O*-(3,4-dihydroxy-2-methylenebutanoyloxy)-6α,15-dihydroxyelema-1,3,11(13)-trien-12-oate (**10**), were isolated from the aerial parts of *Centaurea spinosa*. Nine known flavonoids were also isolated. The structures and the stereochemistry of the new compounds **1** and **2** were deduced by spectroscopic methods. The in vitro activity of **1**−**10** against three Gram-positive and three Gram-negative bacteria was evaluated using a microdilution method, and their in vitro cytotoxic activity was determined against a panel of human tumor cell lines.

Centaurea spinosa L. (Asteraceae), a perennial dwarf shrub with pink florets, is a Greek endemic plant.[1] Characteristic constituents of *Centaurea* species, many of which are used in folk medicine, are elemanolides, eudesmanolides, germacranolides, and guaianolides.[2,3] As a continuation of our research on Greek *Centaurea* species,[4−9] in this paper we report on the chemical profile, as well as the antibacterial and the cytotoxic activities of several sesquiterpene lactones isolated from *C. spinosa*.

A lipophilic extract of the aerial parts of *C. spinosa* was chromatographed on a silica gel column using for elution cyclohexane containing increasing amounts of ethyl acetate and acetone. Further chromatography of the main fractions by silica column chromatography afforded compound **9**[9] and nine known flavonoids, namely, cirsimaritin,[10] salvigenin,[11] desmethoxysudachitin,[10] desmethoxycentauridin,[11] 6,8-dihydroxy-7,4′-dimethylluteolin,[12] 6-hydroxy-7,3′-dimethylluteolin,[11] nepetin,[13] 5,6,8,3′,4′-pentahydroxy-7-methoxyflavone,[12] and retusin.[14] Further purification by reversed-phase HPLC (MeOH−H_2O, 1:1, 2 mL/min) allowed the isolation of the sesquiterpene lactones **1**−**3**,[15] **4**,[5] **5**,[16] **6**,[17] **7**,[18] **8**,[19] and **10**.[19] Compounds **1** and **2** are new natural products. The structures of the known compounds were established by means of 1D and 2D NMR, MS, and UV spectroscopic analysis. The main constituents of *C. spinosa* were sesquiterpene lactones, while flavonoids were isolated in smaller amounts.



Compound **1** showed in its mass spectrum a molecular ion $[M]^+$ at *m/z* 394.1631, compatible with the molecular formula $C_{20}H_{26}O_8$. The IR spectrum exhibited absorption bands typical of hydroxyl (3400 cm^{-1}) and carbonyl groups at 1769 (C=O, γ-lactone), 1724 (C=O, aldehyde), 1700 (C=O, side chain). The ^1H and ^{13}C NMR spectra (Table 1) of compound **1** showed typical signals that suggested a eudesmane framework.[20] The ^{13}C NMR spectrum displayed 20 carbons, which were assigned by HSQC, HMBC, and DEPT 135° experiments as resonances for five quaternary carbons, eight methines, six methylenes, and one methyl carbon atom. The presence of an α-methylene-γ-lactone moiety was confirmed by the ^{13}C NMR signals at δ_C 170.2 (OCO) and 136.6 and 120.2 (C=CH_2). COSY NMR experiments enabled the H-7 signal to be assigned at δ_H 2.88, according to its allylic couplings to H-13a (d, J = 3.2 Hz) and H-13b (d, J = 2.9 Hz). Moreover, the coupling constants between H-7 (tt, J = 10.9/2.9 Hz) and H-8 (dt, J = 4.7/10.8 Hz) indicated a *trans* attachment of the α-methylene-γ-lactone to the decalin ring system.[17] The *trans* configuration of the decalin skeleton was revealed by the coupling constants of H-1, H-4, and H-5 to H-8 and further confirmed by NOESY experiments (Figure 1). NOE signals

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Table 1. ^{1}H and ^{13}C NMR Data and NOESY Correlations for Compounds **1** and **2** (in $CDCl_3$)

	1[a]			**2**[a]		
position	δ_H	NOESY	δ_C	δ_H	δ_C	NOESY
1	3.42 dd (4.3, 10.9)	5, 9b	76.9	3.38 dd (4.9, 11.6)	77.8	4, 5
2a	1.73 m	2b	24.6	1.71 m	27.8	2b
2b	1.56 m	2a		1.64 m		2a
3a	1.83 m	3b	23.1	2.42 m	23.1	3b
3b	1.59 m	3a		1.44		3a
4	2.49 tt (4.0, 11.6)	6, 15	47.8	2.78 t (5.0)	46.0	1, 3a, 5
5	1.87 t (11.6)	1, 7, 15	48.1	2.01 dd (5.6, 12.0)	48.4	7
6	3.97 t (11.1)	4, 8, 14	78.8	4.51 t (11.6)	76.1	8, 14
7	2.88 tt (2.9, 10.9)	5	55.3	2.86 tt (3.3, 10.8)	54.0	1, 5, 6
8	5.28 dt (4.7, 10.8)	6, 9a, 14	69.5	5.31 td (4.2, 10.8)	70.5	6, 9a, 14
9a	2.55 dd (4.5, 12.9)	6, 8, 9b, 14	43.5	2.50 dd (4.2, 12.8)	44.2	6, 8, 9b, 14
9b	1.31 dd (4.8, 13.2)	1, 5, 7, 9a		1.31 (11.2, 12.4)		1, 5, 7, 9a
10			41.9		41.9	
11			136.6		136.6	
12			170.2		169.6	
13a	6.12 d (3.1)	13b	120.2	6.18 d (2.9)	121.2	13b
13b	5.50 d (2.9)	13a		5.63 d (2.9)		13a
14	0.95 s	6, 8, 9a	12.8	0.90 s	15.6	6, 8, 9a
15	9.66 d (3.8)	5	202.2	9.92 s	211.0	6
1′			166.5		165.1	
2′			72.4		130.6	
3′	4.61 dd (3.7, 6.4)	4′a, 5′b	71,5	6.37 ddd (1.6, 3.7, 5.4)	138.6	4′a, 4′b, 5′a, 5′b
4′a	3.83 dd (3.7, 11,1)	4′b	65.9	5.05 dd (4.9, 7.9)	63.3	4′b
4′b	3.59 dd (6.6, 11.1)	4′a		5.05 dd (4.9, 7.9)		4′a
5′a	6.35 s	5′b	127.6	4.30 br s	62.5	5′b
5′b	6.04 s	5′a		4.30 br s		5′a
CH$_3$COO					20.5	
CH$_3$*CO*O				2.08 s	171.1	

[a] The ^{1}H and ^{13}C NMR spectra were measured at 400 and 50.3 MHz, respectively.

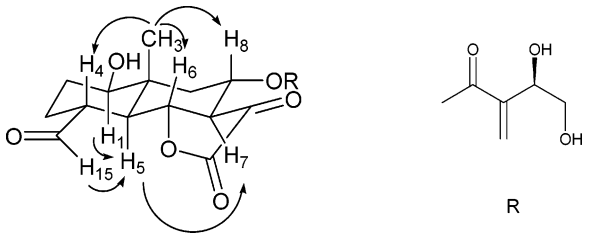

Figure 1. NOESY interactions for compound **1**.

between H-8/CH_3-10 and H-6/CH_3-10 suggested they were on the same side of the molecule (β), while NOE interactions between H-5/H-7 and H-5/H-1 indicated them to be opposite (α). In particular, the lack of a NOE cross-peak between H-5 and CH_3-10 suggested the *trans* fusion of the rings forming the decalin skeleton. H-5 appeared at δ_H 1.87 as a triplet with a coupling constant of 11.6 Hz, showing that this proton has a *trans*-diaxial disposition with H-4 and H-6. This suggested that H-4 is axial, while CHO-15 is equatorial and appeared as a doublet at δ_H 9.66, due to the coupling with H-4. On the basis of NOE interactions between H-3′/H-5′b the relative stereochemistry of the side chain is as shown in Figure 1. Therefore, it was revealed that **1** has a eudesmanolide nucleus with functionality and stereochemistry similar to the previously isolated 8α-*O*-(4-acetoxy-3-hydroxy-2-methylenebutanoyloxy)sonchucarpolide,[5] except for the acyl group of the side chain, since in the ^{1}H NMR spectrum the lack of the sharp singlet at δ_H 2.05 (3H) suggested the absence of the acetate group. A downfield shift was observed for H-4′a at δ_H 3.83 vs 4.29[5] and for H-4′b at δ_H 3.59 vs 4.19.[5] Thus, compound **1** was established as 8α-*O*-(3,4-dihydroxy-2-methylenebutanoyloxy)sonchucarpolide (4-*epi*-malacitanolide).

Compound **2** showed in its mass spectrum a molecular ion $[M]^+$ at *m/z* 436.1716, compatible with the molecular formula $C_{22}H_{28}O_9$. The IR spectrum afforded absorption bands typical of hydroxyl (3400 cm^{-1}) and carbonyl groups [1769 (C=O, γ-lactone), 1748 (C=O, ester) and 1733 (C=O, aldehyde), 1718 cm^{-1} (C=O, side chain)]. The ^{1}H and ^{13}C NMR spectra (Table 1) of compound **2** showed typical signals that suggested a eudesmane framework.[20] The ^{13}C NMR spectrum displayed 22 carbons, which were assigned by HSQC, HMBC, and DEPT 135° experiments to the resonances of six quaternary carbons, eight methines, six methylenes, and two methyl carbon atoms. The presence of an α-methylene-γ-lactone moiety was confirmed by the ^{13}C NMR signals at δ_C 169.6 (OCO) and 136.6 and 121.2 (C=CH_2). The relative stereochemistry of compound **2** was established using *J* values and NOE data derived from its ^{1}H NMR and NOESY spectra. The occurrence of a NOE between H-9b and protons H-7 and H-5 suggested that these protons are oriented on the same side, while H-9a, H-8, and H-6 have the opposite orientation. This was in full agreement with the observed ^{1}H NMR coupling constants. The lack of a NOE cross-peak between H-15 and H-4 suggested that they have a different orientation, resulting in the following differences: H-5 was deshielded at δ_H 2.01 (vs δ_H 1.87 in compound **1**) and CHO-15 is axial and appeared as a singlet deshielded at δ_H 9.92 (vs δ_H 9.66 in compound **1**). The identity of the ester side chain was

Table 2. Minimum Inhibitory Concentrations (MIC, μg/mL) of Compounds **1**−**10**[a]

compound	*S. aureus*	*B. cereus*	*M. flavus*
1	5.0	5.0	0.625
2	3.5		0.437
3	5.0		5.0
4	5.0		5.0
5	5.0	1.25	5.0
6	5.0	1.25	0.625
7	5.0	1.25	5.0
8		1.25	5.0
9	5.0	1.25	5.0
10	5.0		
streptomycin	0.5	0.1	0.1

[a] Compounds **1**−**10** were inactive against tested Gram-negative bacteria.

Table 3. GI_{50}, TGI, and LC_{50} Data (μM) of Compounds **1**−**10** and Vinblastine[a−c]

	1			**5**			**6**			**8**			**10**			vinblastine		
cell line	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LB_{50}	GI_{50}	TGI	LC_{50}
DLD1	*b*	*b*	*b*	*c*	*c*	*c*	*b*	*c*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	<0.01	2.44	2.48
SF268	*b*	*b*	*b*	29.3	*b*	*b*	7.7	38.0	*b*	*b*	*b*	*b*	37.6	*b*	*b*	<0.01	1.64	2.48
MCF7	31.7	*b*	*b*	*b*	*c*	*c*	10.0	*b*	*b*	37.8	*b*	*b*	*b*	*b*	*b*	<0.01	1.60	2.50
H460	32.4	*b*	*b*	*c*	*c*	*c*	37.0	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	<0.01	0.35	2.10
OVCAR3	36.4	*b*	*b*	*c*	*c*	*c*	4.7	*b*	*b*	37.4	*b*	*b*	31.9	*b*	*b*	<0.01	<0.01	0.92

[a] The values represent the means of three independent experiments run in triplicate. SD values never exceeded 15% of the mean value. Compounds **2**−**4**, **7**, and **9** were inactive for all cell lines. [b] Not active at concentrations < 40 μM (maximum concentrations tested). [c] NT, not tested.

deduced from a double double doublet at δ_H 6.37 (1H, J = 1.6, 3.7, 5.4 Hz), which was coupled to a broad double doublet at δ_H 5.05 in the ^{1}H NMR spectrum (2H, J = 4.9, 7.9 Hz). This latter signal was coupled to a broad singlet at δ_H 4.30 (2H). The chemical shifts and the pattern of these signals strongly suggested the presence of a 4′-acetoxy 5′-hydroxyangeloylate moiety.[17] This was confirmed by the signals at δ_C 165.1 (ester carbonyl), 130.6 (C), 138.6 (CH), 63.3 (CH_2), 62.5 (CH_2), 20.5 (CH_3COO), and 171.1 (CH_3*C*OO) in the ^{13}C NMR spectrum. For the acetyl group, the position of C-4′ was assigned on the basis of the HMBC spectrum, due to the observed signal between the carbonyl group and the H-4′ protons. Moreover, the deshielding of H-4′ protons (δ_H 5.05) compared to the H-5′ protons (δ_H 4.30) corroborated this observation. From the above observations, compound **2** was assigned as 8α-*O*-(4-acetoxy-2-hydroxymethylbuten-2-oyloxy)-4-*epi*-sonchucarpolide.

The results of the antibacterial and cytotoxic activities of compounds **1**−**10** are shown in Tables 2 and 3, respectively. Compound **2** showed a moderate inhibitory effect against *S. aureus* and was also active against *M. flavus*. All sesquiterpene lactones tested were found to have a slight inhibitory effect against the Gram-positive bacteria. However, they were inactive against the Gram-negative bacteria in the panel. Among the compounds tested, elemanolide **6**, with an α-methylene-γ-lactone moiety, was the most active against all cell lines and exhibited a significant growth inhibitory activity, below 5 μM, against OVCAR3. This observation is in accordance with previous results.[9] The acetylation of the free OH-4′ of the side chain seems to be important, since compound **8** was found to be more active than **9**. On comparing the activities of elemanolides **5** and **6**, it seems that the presence of an aldehyde group enhances the cytotoxic activity of sesquiterpene lactones.

Experimental Section

General Experimental Procedures. Optical rotation values were measured at 20 °C in $CHCl_3$ (Uvasol) on a Perkin-Elmer 341 polarimeter. IR spectra were obtained on a Perkin-Elmer Paragon 500 FT-IR spectrophotometer. The ^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (50.3 and 100.6 MHz) were recorded using Bruker DRX 400 and Bruker AC 200 spectrometers. Chemical shifts are reported in δ (ppm) values relative to TMS. COSY, HMQC, HSQC, HMBC, and NOESY (mixing time 950 ms) NMR spectra were performed using standard Bruker microprograms. Mass spectrometric data were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, South Bend, IN. HR/EM FABMS data were recorded on a double sector JEOL JMS-AX505HA mass spectrometer. Vacuum-liquid chromatography (VLC):[21] silica gel (Merck; 43−63 μm). Column chromatography: silica gel 60H SDS (40−63 μm), gradient elution with the solvent mixtures indicated in each case. Reversed-phase chromatography: CE 1100 liquid chromatography. HPLC support: Preparative HPLC was performed using a C_{18} 25 cm × 10 mm Techsil 10 column. Fractionations were monitored by TLC on silica gel 60 F-254, Merck art. 5554; Cellulose, Merck art. 5716; RP 18 F-254, Merck, art. 5685 with visualization under UV light (254 and 365 nm) and with anisaldehyde-sulfuric acid reagent on silica gel and Neu's reagent on cellulose.[22]

Plant Material. The aerial parts of *C. spinosa* were collected at Lagonisi (Attiki, Central Greece) in June 2000. The plant was authenticated by Dr. T. Constantinidis (Institute of Systematic Botany, Agricultural University of Athens), and a voucher specimen was deposited in the Herbarium of the Institute of Systematic Botany, Agricultural University of Athens (Constantinidis 9135).

Extraction and Isolation. The fresh aerial parts of *C. spinosa* (0.79 kg) were finely ground and extracted at room temperature with cyclohexane−Et_2O−MeOH (1:1:1). The extract was washed with brine, the aqueous layer re-extracted with EtOAc, and the organic layer dried with Na_2SO_4 and concentrated under reduced pressure. The residue (32.5 g) was fractionated by VLC on silica gel (10.0 × 6.0 cm), using cyclohexane−EtOAc−Me_2CO mixtures of increasing polarity as eluents, to give nine fractions of 500 mL each. Fraction D (cyclohexane−EtOAc, 25:75, 3.27 g) was subjected to further VLC on silica gel (5.0 × 10.0 cm; cyclohexane−EtOAc−MeOH, 10:0:0 to 0:0:10), which led to the isolation of **9** (354 mg), **11** (11 mg), **12** (32 mg), **13** (1.7 mg), **14** (4.9 mg), **15** (1.2 mg), **16** (2.1 mg), **17** (37 mg), **18** (1.1 mg), and **19** (2.6 mg). Further purification by reversed-phase HPLC (MeOH−H_2O, 1:1, 2 mL/min) resulted in the isolation of **2** (1.5 mg), **4** (1.4 mg), **6** (4.1 mg), and **7** (3.8 mg); t_R 17.2, 17.8, 27.2, and 48.5 min, respectively. VLC on silica gel (10.0 × 5.0 cm; cyclohexane−EtOAc−MeOH, 10:0:0 to 0:0:10) of fraction E (EtOAc 100%; 9.45 g) afforded 19 fractions. Fraction 10 (28.4 mg) of the latter VLC was subjected to reversed-phase HPLC (MeOH−H_2O, 1:1, 2 mL/min) and allowed the isolation of **2** (2.2 mg) and **5** (2.6 mg); t_R 16.9 and 14.0 min, respectively. VLC of fraction 13 (7.83 g) followed by reversed-phase HPLC (MeOH−H_2O, 1:1, 2 mL/min) yielded **3** (1.3 mg) and **8** (25 mg); t_R 16.5 and 19.3 min, respectively. Fraction G (EtOAc−Me_2CO, 75:25; 791.6 mg) was subjected to column chromatography on silica gel (17.0 × 3.0 cm; CH_2Cl_2−MeOH, 10:0 to 1:1) and afforded nine fractions. Fraction 6 (0.38 g) was subjected to column chromatography over silica gel (19.0 × 1.8 cm; CH_2Cl_2−MeOH, 10:0 to 1:1) and yielded eight fractions. Further purification of fraction 5 (80 mg) by reversed-phase HPLC (MeOH−H_2O, 1:1, 2 mL/min) allowed the isolation of **1** (8.5 mg) and **10** (21 mg); t_R 10.0 and 18.4 min, respectively.

8α-*O*-(3,4-Dihydroxy-2-methylenebutanoyloxy)sonchucarpolide (1): oil; $[\alpha]^{20}_D$ +28.2° (*c* 0.17, $CHCl_3$); IR (KBr) ν_{max} 3600−3300, 1769, 1724, 1700 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HR/EM FABMS *m/z* $[M]^+$ 394.1631, calcd for $C_{20}H_{26}O_8$, 394.1628.

8α-*O*-(4-Acetoxy-2-hydroxymethylbuten-2-oyloxy)-4-*epi*-sonchucarpolide (2): oil; $[\alpha]^{20}_D$ +48.4° (*c* 0.05, $CHCl_3$); IR (KBr) ν_{max} 3600−3300, 1769, 1769, 1748, 1733, 1718 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HR/EM FABMS *m/z* $[M]^+$ 436.1716, calcd for $C_{22}H_{28}O_9$, 436.1733.

Bioassays. The compounds were dissolved at 10 mg/mL with DMSO and diluted with the nutrient medium to a concentration of 1000 μg/mL. Final concentrations of 50.0, 25.0, 12.5, and 6.25 μg/mL were used. The proportion of DMSO

never exceeded 1% in the medium.[23] The following organisms were used. Gram-positive: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolates), *Micrococcus flavus* (ATCC 10240). Gram-negative: *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (clinical isolates), *Escherichia coli* (ATCC 35218).

Bacterial species were cultured overnight at 37 °C in TSB (tryptone soya broth, Oxoid CM 129). Suspensions contained ca. 10^9 cells/mL. The antibacterial assays were carried out by the microdilution method.[24,25] Suspensions were adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. Dilutions of the inocula were subcultured on TSA (tryptone soya agar, Oxoid CM 131) to verify the absence of contamination and to check the validity of the inoculum. Minimum inhibitory concentration (MICs) determination was performed by a serial dilution technique using 96-well microtiter plates. The plates were incubated for 36 h at 37 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). DMSO was used as a control, while streptomycin was used as a positive control.

The cytotoxic activity of compounds **1**−**10** was tested against DLD1 (colon), SF268 (CNS), MCF7 (breast), H460 (non-small-cell lung cancer), and OVCAR3 (ovarian) cell lines and determined by the sulfurhodamine B (SRB) assay, as previously described.[26] The data represent the mean of three experiments in triplicate and were analyzed using a two-tailed Student's *t*-test. The following parameters were determined through our own customized software: GI_{50}, TGI, and LC_{50}.[27] Briefly, GI_{50} is the concentration where $100 \times (T - T_0)/(C - T_0) = 50$ and measures the growth inhibitory potency of the tested compound. TGI is the concentration of the test compound where $100 \times (T - T_0)/(C - T_0) = 0$ and measures the cytostatic effect of the compound. T is the optical density of the test well after a 48 h period of exposure to the test compound; T_0 is the optical density of the cell population at time zero (when the compound is added), and C is the optical density of the control well, where cells were incubated for 48 h, and represents the cytotoxic activity of the compound. LC_{50} is the concentration of the test compound, where $(T/T - T_0) \times 100 = -50$.

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