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SYNTHESIS OF THE NOVEL CAGE AMIDES AND IMIDES AND EVALUATION OF THEIR ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY

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Cage amides and imides bearing bicyclo[2.2.1]- and bicyclo[2.2.2]-subunits were synthesized and evaluated both for antimicrobial activity toward five key ESKAPE pathogenic bacteria: one Gram-positive bacteria methicillin-resistant Staphylococcus aureus (ATCC 43300), four Gram-negative bacteria Escherichia coli (ATCC 25922), Klebsiella pneumoniae (ATCC 700603), Acinetobacter baumannii (ATCC 19606) and Pseudomonas aeruginosa (ATCC 27853) and for antifungal activity towards pathogenic fungal strains Candida albicans (ATCC 90028) and Cryptococcus neoformans var. Grubii (H99; ATCC 208821). Compound VP-4539 with bicyclo[2.2.2]octene motif demonstrated the highest cytotoxic activity towards C. neoformans, while human keratinocytes of HaCaT line, murine fibroblasts of Balb/c 3T3 line and mitogen-activated lymphocytes of peripheral human blood were found to be tolerant to its action. ***VP-4539*** *compound did not intercalate into salmon sperm DNA indicating that its cytotoxicity is not related to intercalation into nucleic acid.*

K e y w o r d s: cage compounds, bicyclo[2.2.1]heptane, bicyclo[2.2.2]octene, antimicrobial, antifungal, cytotoxicity, DNA intercalation, human keratinocytes, lymphocytes.

In recent years, infectious diseases have become a major challenge to the global health system as killing millions of people worldwide. In addition, they affect socio-economic stability, contributing not only to the growth of the disease's cases, but also leading to psychological disorders, reduction of the economy, and standard of living as a whole [1]. A wide variety of pathogens and the constant emergence of new multidrug-resistant pathogenic strains complicate the treatment and prevention of infectious diseases. Invasive fungal infections represent a global problem resulting in 1.7 million deaths every year. They are common in immunocompromised patients, as reflected in their chemotherapy, acquired immune deficiency syndrome, and/or organ transplantation [2]. The recent annual incidence of invasive aspergillosis, candidiasis, and mucormycosis is over 300,000, 750,000, and 10,000 cases, respectively [3]. Therefore, to address the above problems there is an urgent need to develop new anti-bacterial and anti-fungal drugs.

The chemistry of organic polycyclic cage compounds has intrigued medicinal chemists for over 70 years, yet little is published about their pharmacological profiles. Compounds with unusual three-dimensional structures have frequently attracted the attention of chemists as possible synthetic targets because the shape of chemical structures in drug discovery is a crucial component for evoking molecular recognition events with biological targets [4]. Medicinal chemists typically exploit novel conformationally restricted 3D-shaped building blocks with a high fraction of C(sp^3)-hybridized carbons (Fsp^3) [5]. Polycyclic cage compounds have important pharmaceutical applications, ranging from the symptomatic and proposed curative treatment of neurodegenerative diseases such as Parkinson's and Alzheimer's disease, to use as antiviral agents against influenza and the immunodeficiency virus (HIV) (Fig. 1). The polycyclic cage appears to be a useful scaffold to yield drugs with a wide scope of applications, and can be used also to modify and

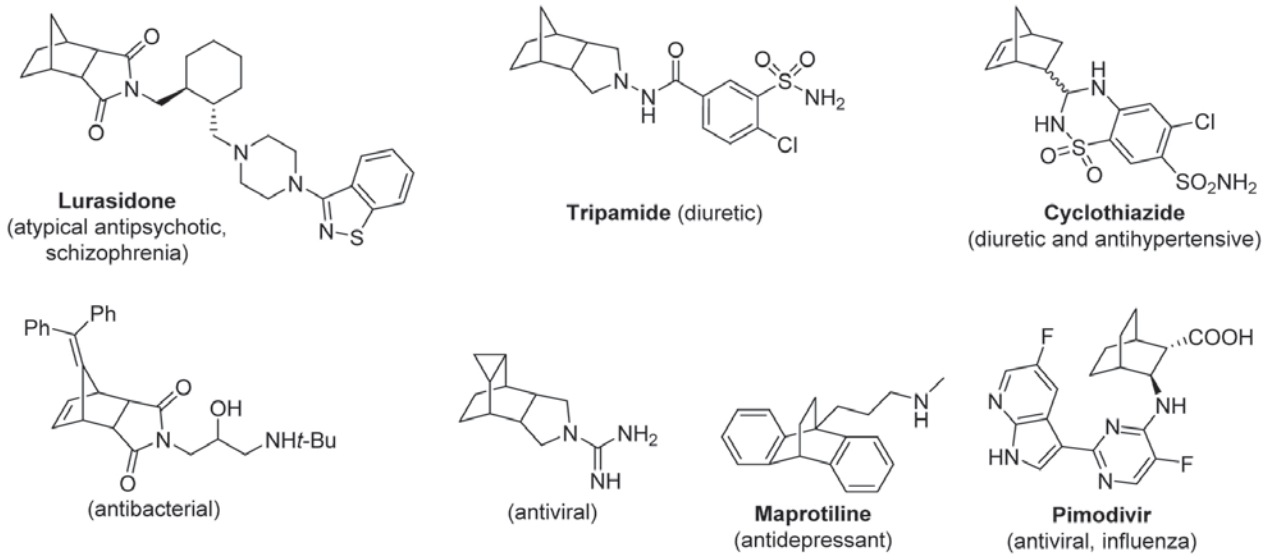


Fig. 1. A representative polycyclic cage-like drugs and bioactive compounds bearing bicyclo[2.2.1] and bicyclo[2.2.2] motives

improve the pharmacokinetic and pharmacodynamic properties of drugs in current use [6].

The synthesis, chemistry and pharmacology of polycyclic cage-like compounds have attracted considerable attention in recent years both from our group [7-11] and many others. It is not surprising, therefore, that currently, medicinal chemists are increasingly searching for novel, unique, 3D-shaped, and conformationally restricted screening compounds. Thus, cage-like molecules are attractive for the drug discovery in terms of modern medical chemistry and new concepts, and therefore we set out to design and synthesize such derivatives that would be of interest as potential anti-bacterial and anti-fungal agents. The value of bridged ring systems in medicinal chemistry has increasingly been recognized, for example as isosteres of phenyl and piperidine rings [12]. It has been shown that for some drug candidates, replacing a phenyl group with bicyclo[2.2.2]octanes could maintain pharmacological efficacy while improving solubility and oral bioavailability [13]. Recently, we found that the thieno[2,3-*d*]pyrimidin-4(3*H*)-one bearing bicyclo[2.2.1]heptane motif possessed high toxicity toward human leukemia HL-60, cervix carcinoma KB3-1, and colon carcinoma HCT116 cells [14]. It was reported that 2,2′-bis(bicyclo[2.2.1]heptane) caused DNA damage in *E. coli* cells through the generation of superoxide anion and hydrogen peroxide [15], while the toxicity of metal-salen complexes based on 1,2-bicyclo[2.2.2]octane structure toward human colon carcinoma cells of HCT116 line was not accompanied by their intercalation into DNA molecule [16].

Materials and Methods

All chemicals were of laboratory grade and used without further purification. The compounds were synthesized according to previously described synthetic procedures (**VP-453**9 [17-19], **VP-4557** [17-19], **VP-4536** [20], **VP-4521** [21], **VP-4511** [22], **VP-4510** [23]). The full compound characterizations are given in cited papers. The synthesis of new compounds is given below.

2-((*3aR,4S,7R,7aS*)-1,3-Dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisoindol-2-yl)-N,N-diethylacetamide (VP-4538). To a solution of 1.64 g (10 mmol) Endic anhydride 3 in 20 ml of acetic acid 0.75 g (10 mmol) glycine was added. The obtained mixture was heated under reflux for 6 h, concentrated *in vacuo* and triturated with water (30 ml). The product was filtered, washed with cold water and dried (88%, 1.94 g). The obtained acid (1.94 g, 8.8 mmol) was dissolved in 50 ml of DCM. Under cooling to 0°C 2.01 g (9.67 mmol) PCl_5 was added in portions. The reaction mass was allowed to warm to room temperature and mixed for 6 h. After concentration *in vacuo*, the residue was triturated with 100 ml of hexane under cooling. The corresponding chloroanhydride was filtered and

dried (2.20 g, 95%). To a solution of 2.20 g (9.18 mmol) this chloroanhydride in 50 ml of dry DCM 1.34 g (18.4 mmol) diethylamine was added dropwise under cooling to 0°C. The reaction mass was allowed to warm to room temperature and mixed for 4 h, washed with brine (2×50 ml), dried over sodium sulfate and concentrated *in vacuo* to get a crude product. The obtained amide was recrystallized from 2-propanol. Yield: 2.25 g (89%) as a white solid; mp 136–138°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.03 (s, 2H), 4.05 (s, 2H), 3.41 (s, 2H), 3.24 (s, 2H), 2.96 (q, 2H, J 7.0 Hz), 2.78 (q, 2H, J 7.0 Hz), 1.08 (t, 6H, J 7.0 Hz). MS (*m/z*, APCI) 277.3 (M^+ + 1); Anal. calcd for $C_{15}H_{20}N_2O_3$: C, 65.20; H, 7.30; N, 10.14. Found: C, 64.91; H, 7.11; N, 10.38.

Methyl 4-((*3aR,4R,7S,7aS*)-1,3-dioxooctahydro-2H-4,7-methanoisoindol-2-yl)benzoate (VP-4540). To a solution of 1.64 g (10 mmol) Endic anhydride 3 in 20 ml of acetic acid 1.37 g (10 mmol) 4-aminobenzoic acid was added. The obtained mixture was heated under reflux for 6 h, concentrated *in vacuo* and triturated with water (30 ml). The product was filtered, washed with cold water and dried (91%, 2.58 g). The obtained acid (2.58 g, 9.1 mmol) was dissolved in 50 ml of DCM. Under cooling to 0°C, 2.09 g (10.02 mmol) PCl_5 was added in portions. The reaction mass was allowed to warm to room temperature and mixed for 6 h. After concentration *in vacuo*, the residue was triturated with 100 ml of hexane under cooling. The corresponding chloroanhydride was filtered and dried (2.66 g, 97%). To a solution of 2.66 g (8.83 mmol) this chloroanhydride in 50 ml of dry methanol was refluxed for 1 h and concentrated *in vacuo* to get a crude product. The obtained ester was recrystallized from 2-propanol. Yield: 2.23 g (85%) as a white solid; mp 165–167°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.01 (d, J 7.9 Hz, 1H), 7.82 (s, 1H), 7.67 (t, J 7.9 Hz, 1H), 7.54 (d, J 7.9 Hz, 1H), 3.88 (s, 3H), 3.29 (s, 2H), 2.69 (s, 2H), 1.69 (d, J 9.7 Hz, 1H), 1.59 (м, 3H), 1.30 (м, 2H). MS (*m/z,* APCI) 298.2 (M^+ + 1); Anal. calcd for $C_{17}H_{15}NO_4$: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.81; H, 5.31; N, 4.50.

(*1S,3R*)-3-((4-Bromophenyl)carbamoyl)cyclopentane-1-carboxylic acid (VP-4543). To a solution of 1.40 g (10 mmol) anhydride 5 in 20 ml of benzene, 1.72 g (10 mmol) 4-bromoaniline was added. The obtained mixture was vigorously stirred for 2 h and precipitate was filtered and recrystallized from 2-propanol. Yield: 2.75 g (88%) as a white solid; mp 151–153°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.05 (s, 1H), 9.97 (s, 1H), 7.57 (d, J 8.6 Hz, 2H), 7.45 (d, J 8.6 Hz, 2H), 2.87–2.67 (m, 2H), 2.13 (m, 1H), 1.97–1.77 (m, 5H). MS (*m/z*, APCI) 312.0 and 314.0 (M^+ + 1); Anal. calcd for $C_{13}H_{14}BrNO_3$: C, 50.02; H, 4.52; N, 4.49. Found: C, 50.19; H, 4.31; N, 4.59.

(*1R,2S,3R,4S*)-3-(o-Tolylcarbamoyl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid (VP-4520). To a solution of 1.64 g (10 mmol) Endic anhydride 3 in 20 ml of benzene, 1.07 g (10 mmol) 2-methylaniline was added. The obtained mixture was vigorously stirred for 12 h and precipitate was filtered and recrystallized from 2-propanol. Yield: 2.13 g (84%) as a white solid; mp 160–162°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.59 (s, 1H), 9.02 (s, 1H), 7.36 (d, J 7.7 Hz, 1H), 7.16 (d, J 7.1 Hz, 1H), 7.10 (t, J 7.3 Hz, 1H), 7.02 (t, J 7.1 Hz, 1H), 6.20 (m, 1H), 6.06 (m, 1H), 3.46 (m, 1H), 3.24 (m, 1H), 3.10 (m, 1H), 3.01 (m, 1H), 2.19 (s, 3H), 1.34 (d, J 7.7 Hz, 1H), 1.29 (d, J 7.7 Hz, 1H). MS (*m/z*, APCI) 272.1 (M^+ + 1); Anal. calcd for $C_{16}H_{17}NO_3$: C, 70.83; H, 6.32; N, 5.16. Found: C, 70.69; H, 6.49; N, 4.98.

Antimicrobial assays via CO-ADD [24]

The compounds were investigated for activity towards one Gram-positive bacteria (*Staphylococcus aureus* ATCC 43300 MRSA), four Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Acinetobacter baumannii* ATCC 19606), and fungi (*Candida albicans* ATCC 90028 and *C. neoformans* H99 ATCC 208821), and this research was performed by the Community for Open Antimicrobial Drug Discovery (CO-ADD).

Initially, the tests were carried out at a single compound concentration of 32 μg/ml in duplicate, to identify any active compound. All substances were dissolved in dimethylsulfoxide (DMSO) to form a stock concentration of 10 mg/ml. Aliquots were diluted in water and 5 μl were dispensed into empty 384-well plates in duplicate for each strain and cell-assayed. As soon as cells were added to the plates, this gave a final compound concentration of 32 μg/ml, a maximum DMSO concentration of 0.3%. DMSO in such concertation did not show inhibition effect on the growth of studied strains.

All bacteria were overnight cultured in cation-adjusted Q14 Mueller–Hinton broth (CAMHB) at 37°C. The resultant mid-log phase cultures were added to each well of the compound-containing plates (384-well nonbinding surface plates-Corning 3640), giving a cell density of 5×10^5 CFU/ml (colony-

forming units/ml). All plates were covered and incubated at 37°C for 18 h without shaking. Inhibition of bacterial growth was determined by measuring absorbance at 600 nm. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. Growth inhibition of *C. albicans* was determined by measuring absorbance at 530 nm, while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm, after the addition of resazurin (0.001% final concentration) and incubation at 35°C for additional 2 h. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. Percentage growth inhibition of an individual sample is calculated based on negative controls (media only) and positive controls (bacterial/fungal media without inhibitors). Negative inhibition values indicate that the growth rate (defined in OD = 600 nm) is higher compared to the negative control (Bacteria/fungi only, set to 0% inhibition). The growth rates for all bacteria and fungi have a variation of -/+ 10%, which is within the reported normal distribution of bacterial/fungal growth. Any significant variation (or outliers/hits) is identified by the modified Z-Score, and actives are selected by a combination of inhibition value and Z-Score. Growth inhibition was evaluated as a percentage between untreated cells (positive growth control) and media only (negative growth control). Compounds with ≥80% growth inhibition were selected as active compounds in the initial screening, and MIC was determined following EUCAST recommendations. Also, 80% growth inhibition was used as a threshold for full inhibition.

CFU (Colony forming units) assay on *C. albicans* strains

In this work, two other strains of fungi – *C. albicans* ATCC 885-655 – a classic laboratory strain obtained from ATCC, and *C. albicans* N12 clinical isolate obtained from the Department of Genetics and Biotechnology of Ivan Franko Lviv National University were used. This strain is resistant to itraconazole, ketonazole, clotrimazole, fluconazole.

A suspension of *Candida* strains containing 10^7 cells/ml was prepared by suspending cells taken from the colonies grown on the Sabouraud agar, pH 5.8. Cells number was counted in Horyaev hemocytometric camera, since the size of *Candida* cells (2.5-4 μm) permitted doing that accurately. The tested compound solution in 10, 5 and 2.5 μl volume was introduced into 3 round bottom Eppendorf tubes, and thereafter, 100 μl of *Candida* cells suspension was added. Two control tubes were prepared: at the start (time 0) and at the end (4 h) of incubation. The tubes were incubated for 4 h at 37°C (except control 0 kept at 4°C). 10 μl aliquote was withdrawn at the end of incubation from each tube after thorough mixing, diluted 10,000-fold with water and 0.2 ml of this dilution was distributed on the surface of Sabouraud agar medium, pH 5.8, in the Petri dish. They were incubated at 37°C and after distinct formation of colonies (usually after 24 h) the image was scanned and colonies were counted with the aid of the Photoshop program. The number of colonies in control tube at 0 h time must be 200 ± 50 per dish, in control after 4 h of incubation colonies number must be 1.5-2.5-fold higher. The experiment was abolished when the increase in number of colonies in control after the incubation was less than 1.5-fold. The effect of compound samples on the viability of *Candida* cells was expressed as a ratio of colony number.

The index lower than control (0 hour) was considered as candidacidal effect, while higher than control (0 hour) but less than in control after 4 h of incubation was classified as growth inhibition, and higher than in control after 4 h of incubation indicated a stimulation of growth [25]. DMSO in such low concertation did not show inhibition effect on the growth of *Candida* strains.

Cells culture and cytotoxicity MTT assay

Human keratinocytes of HaCaT line and murine fibroblasts of Balb/c 3T3 line were obtained from a Collection at the Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine (Kyiv, Ukraine).

Cells were grown in the DMEM (Biowest, Nuaille, France) culture medium supplemented with 10% of fetal bovine serum (Biowest, Nuaille, France) under standard conditions. *In vitro* evaluation of cytotoxic activity of the synthesized compounds in comparison with doxorubicin, used as a reference control, towards HaCaT and Balb/c 3T3 cells was measured by the MTT test [26]. Briefly, cells were seeded for 24 h in 96-well microtiter plates at a concentration of 5,000 cells/well (100 μl/well); after that, cells were incubated for 72 h with various additions of the synthesized compounds or DMSO (1; 10; 100;

1,000; 2,500 μM), or Dox (1; 10 μM). MTT, which is converted to dark blue, water-insoluble formazan by the mitochondrial dehydrogenases, was used to determine viable cells according to the Sigma-Aldrich protocol. Formazan was dissolved in DMSO, and the results of the reaction were determined by an Absorbance Reader BioTek ELx800 (BioTek Instruments, Inc., Winooski, VT, USA).

The study protocol with human lymphocytes isolated from healthy adult human peripheral blood was approved by the Ethics Committee of the Institute of Cell Biology of National Academy of Sciences of Ukraine (protocol #2 dated by January 27, 2019). Lymphocytes of human peripheral blood were isolated from blood consisting of anti-coagulant sodium heparin solution 10 U/ml (B. BRAUN MEDICAL, S.A., Spain) on density gradient of Gradisol G (Polfa, Poland), as described [27]. The blood: Gradisol G mixture (1:1) was centrifuged at 400×g at room temperature for 30 min. The cells were washed in phosphate-buffered saline (PBS). The residual erythrocytes were removed from lymphocytes population by the hypotonic lysis. Lymphocytes were cultured in the RPMI-1640 (Biowest, France) medium supplemented with 20% fetal bovine serum (Biowest, France) at 95% air and 5% CO_2, and 37°C. The lymphocytes were activated using phytohemagglutinin-L (PHA-L, 1 μg/ml, Sigma-Aldrich, USA) mitogen and incubated for the next 24 h before treatment with studied compounds.

The evaluation of the anti-proliferative activity *in vitro* of the studied compounds or DMSO (1; 10; 100; 1,000; 2,500 μM), or Dox (1; 10 μM) towards mitogen-activated lymphocytes (100,000 cells/100 μl) of human peripheral blood was conducted on 48 h using MTT assay (EZ4U, Biomedica, Vienna, Austria). The optical density was measured with the Absorbance Reader at 490 nm with 630 nm as a reference wavelength. The reduction of cell growth (in percentages, %) was calculated as ratio of absorbance in treated cells relative to absorbance in control cells. The anti-proliferation activity of the studied compounds was expressed as the half maximal inhibitory concentration IC_{50} (the concentration of sample that reduces the 50% of cell growth).

DNA intercalation assay using methyl green

DNA intercalation assay using methyl green was conducted as described previously [28]. Briefly, 485 μl of salmon sperm DNA (50 μg/ml, Sigma-Aldrich, USA) were incubated for 1 h at 37°C with 15 μl of methyl green solution (1 mg/ml, Sigma-Aldrich, USA). 500 μl of compound **VP-4539** (1; 10; 100 μM), doxorubicin (1, 10 μM, Actavis, Romania), ampicillin (1; 10 μM) were added to methyl green-DNA complex and incubated for 2 hrs at 37°C in the dark. Ethydium bromide (EtBr, 1 and 10 μg/ml) was used as a positive control. Absorption of methyl green was measured at 630 nm using a fluorescence plate reader (Absorbance Reader BioTek ELx800, BioTek Instruments, Inc., Winooski, Vermont, USA).

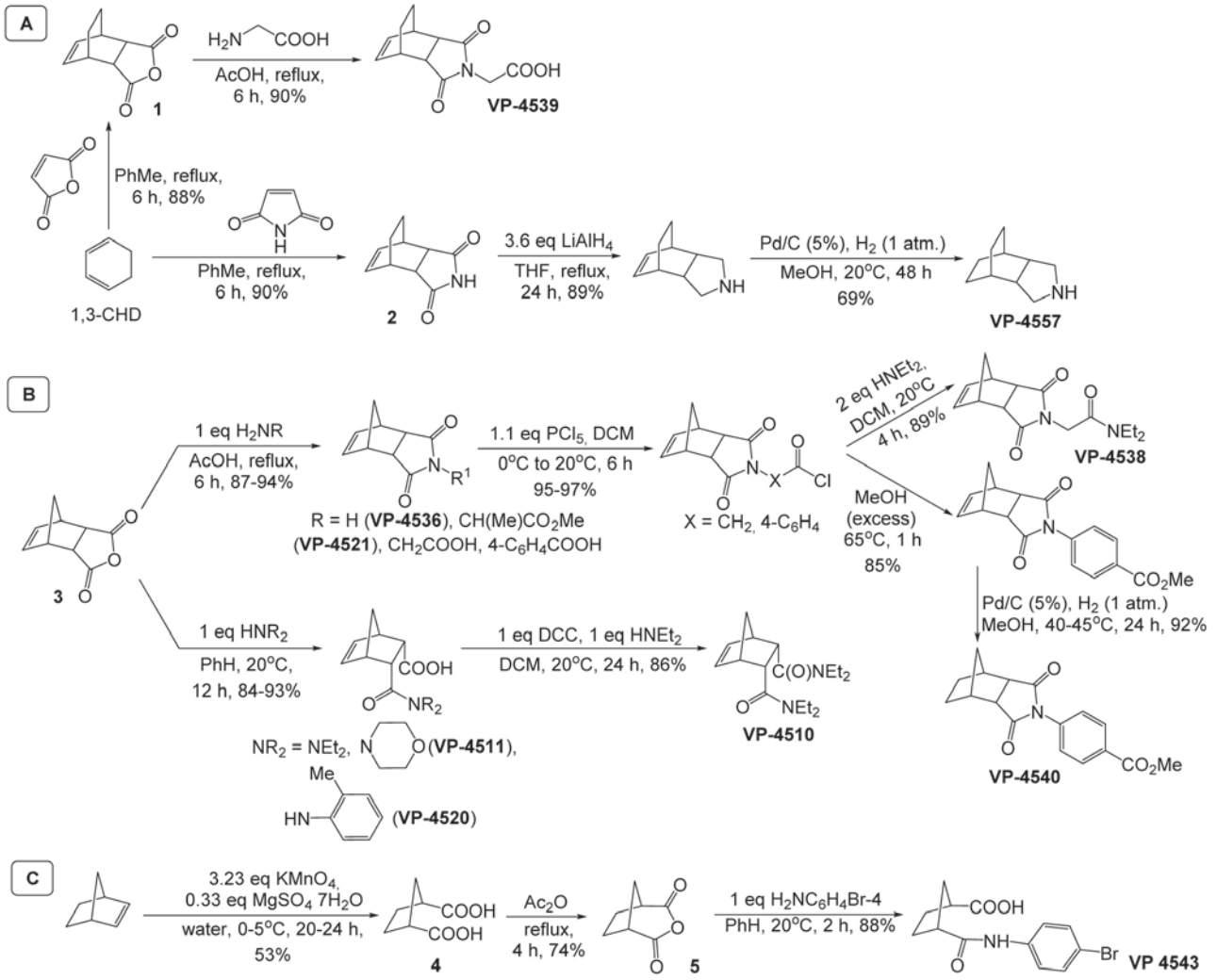
Statistical analysis. Z-Score analysis was done to investigate outliers or hits among the samples. The Z-Score was calculated based on the sample population using a modified Z-Score method which accounts for possible skewed sample population. The modified method uses median and median average deviation (MAD) instead of average and Standard deviation (SD), and a scaling factor: M(i) = 0.6745*(x(i)–median(x))/MAD). All screening was performed as two replicas (n = 2), with both replicas on different assay plates, but from single plating and performed in a single screening experiment (microbial incubation). Two values were used as quality controls for individual plates: Z-Factor = 1 – [3*(SD(Negative controls) + SD (Positive Controls))/(average(Positive Controls)-average(Negative controls))].

Cytotoxicity data are presented as the mean ± SD. Results were analyzed and illustrated with GraphPad Prism (version 6; GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using two-way ANOVA with Dunnett's multiple comparisons test (cell growth inhibition). A P-value of <0.05 was considered statistically significant.

Results and Discussion

Chemistry. The preparation of the target compounds **VP-4539**, **VP-4557**, **VP-4536**, **VP-4538**, **VP-4521**, **VP-4540**, **VP-4510**, **VP-4511**, **VP-4543** and **VP-4520** is described in Scheme 1.

Starting from 1,3-cyclohexadiene (1,3-CHD) we synthesized anhydride 1 and imide 2 using Diels-Alder chemistry. Based on these building blocks we got target compounds **VP-4539** and **VP-4557** as previously reported [17-19] (Scheme 1, A). Using readily available endic-anhydride 3 we synthesized imides **VP-4536** [20], **VP-4521** [21], **VP-4538** [22], **VP-4540** [10] and amides **VP-4511** [23], **VP-4510** [29], **VP-4520** of bicyclo[2.2.1]heptane(ene) series by applying common reactions (Scheme 1, B). Amidoacid **VP-4543** was prepared [30] and used for bio-scree-



Scheme 1. Synthetic routes to the target compounds

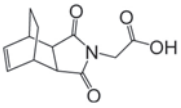
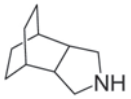
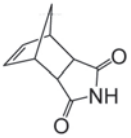
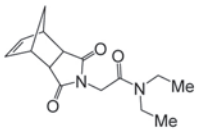
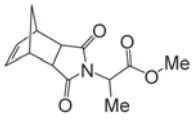
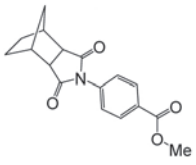
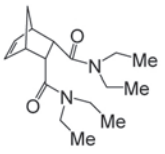
ning for comparison with cage analog. The synthetic route starts with oxidation of norbornene by potassium permanganate in aqueous media [31, 32] followed by cyclization of the resulted *cis*-di-acid **4** into **5** and opening this anhydride with 4-bromoaniline (Scheme 1, C).

Antimicrobial screening. The results of the preliminary screening (two parallel trials) of the newly synthesized compounds **VP-4539**, **VP-4557**, **VP-4536**, **VP-4538**, **VP-4521**, **VP-4540**, **VP-4510**, **VP-4511**, **VP-4543** and **VP-4520** in concentration 32 μg/ml (100–200 μM) on seven pathogens (*S. aureus, E. coli, K. pneumoniae, P. aeruginosa, A. baumannii, C. albicans* and *C. neoformans*) are presented in Table 1.

Most of the tested compounds did not show activity against resistant strains. However, compound **VP-4539** had a highly selective effect on the fungal strain *C. neoformans* (ATCC 208821). At a concentration of 136 μM (32 μg/ml), the compound completely inhibited growth of *C. neoformans.* To confirm the selectivity of action of **VP-4539**, compound was additionally studied toward *C. albicans* ATCC 885-655 (laboratory strain) and *C. albicans* N12 (multidrug-resistant strain) (Table 2).

The obtained data indicate a pronounced fungicidal activity of compound **VP-4539**, at a concentration of 2.5 mM against the laboratory strain of *C. albicans*, as well as at a concentration of 2.5 and 1 mM against its multidrug-resistant strain N12. Fungistatic activity was also observed at a concentration of 100 μM toward both strains. At the same time, at 100 μM compound **VP-4539** inhibited growth of *C. albicans* only by 37.1% of ATCC 885-655 and

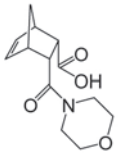
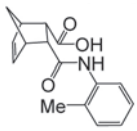
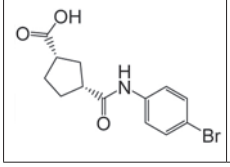
Table 1. Preliminary screening of selected cage amides and imides

Compounds	Growth inhibition in concentration 32 μg/ml, %						
	Bacteria					Fungi	
	S. aureus ATCC 43300	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 700603	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 19606	*C. albicans* ATCC 90028	*C. neoformans* ATCC 208821
VP-4539 	11.75±5.59[a]	-0.30±2.12	1.70±1.20	-0.25±4.03	15.65±0.35	7.50±1.27	**93.30±8.34**
VP-4557 	11.30±1.83	-1.35±0.07	7.05±11.10	-6.75±12.23	7.50±0.28	4.90±0.71	1.55±1.63
VP-4536 	13.90±9.47	-2.30±1.27	1.10 ± 1.70	-0.60±3.68	-5.45±5.16	4.85±0.50	-6.70±5.80
VP-4538 	7.90±3.68	-2.60±2.55	1.90 ± 0.99	-0.70±2.97	11.75±4.17	7.85 ± 2.19	0.45±1.63
VP-4521 	4.30±2.69	-1.10±0.28	4.35±6.15	7.35±4.31	2.00±1.83	2.01±1.83	0.45±1.63
VP-4540 	-4.60±13.29	3.55±2.33	7.15±2.05	8.05±2.48	5.95±13.36	8.75±2.48	12.35±7.28
VP-4510 	-12.20±2.69	-7.15±2.05	-4.05±2.05	-0.75±2.76	-3.35±11.67	6.8 ±5.37	-10.05±5.02

22.1% of N12 strains. This indicates the selectivity of the action of **VP-4539** on *C. neoformans.*

Cytotoxicity for normal cells. To determine the potential cytotoxicity of compound **VP-4539** it was tested on human keratinocytes of HaCaT line, murine fibroblasts of Balb/c 3T3 line and mitogen-activated lymphocytes. Compound does not reach the IC_{50} value for HaCaT and Balb/c 3T3 cells up to 2.5 mM. At the highest dose of 2.5 mM, compound **VP-4539** inhibited the growth of HaCaT cells by 44.0%, of Balb/c 3T3 cells – by 36.7%. Solvent DMSO demonstrated similar toxicity towards HaCaT and Balb/c 3T3 cells. It was found 38.9% and 46.9% of growth inhibition of HaCaT and Balb/c

Table 1. (Continuation)

Compounds	Growth inhibition in concentration 32 μg/ml, %						
	Bacteria					Fungi	
	S. aureus ATCC 43300	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 700603	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 19606	*C. albicans* ATCC 90028	*C. neoformans* ATCC 208821
VP-4511 	0.90±9.62	-0.90±4.95	-2.55±3.32	0.05±0.78	0.60±2.12	6.40±0.85	-13.85±4.60
VP-4520 	16.8±1.41	2.00±4.67	3.65±1.06	3.70±0.71	16.40±2.69	4.25±0.78	-8.05±1.49
VP-4543 	-20.35±5.87	3.75±1.77	7.30±1.41	3.75±2.76	17.75±3.18	10.00±0.14	18.40±11.17

Note: Data are presented as mean ± SD calculated from two parallel trials [a]

Table 2. Antifungal activity of VP-4539 against C. albicans ATCC 885-655 (laboratory strain) and C. albicans N12 (multidrug-resistant strain)

Concentration	Relative percentage of CFU[#]			
	ATCC 885-655		N12	
	% to C0	% to C4	% to C0	% to C4
C0	100.0±3.7		100.0±3.2	
C4	347.5±3.9	100.0±3.9	337.0±3.5	100.0±3.5
VP-4539, 1 μM	335.4±7.3	96.5±5,1	332.2±5.8	98.6±5.8
VP-4539, 10 μM	302.0±6.5	86.9±6.5	330.8±5.5	98.2±5.5
VP-4539, 100 μM	218.7±5.9	62.9±5.9	262.6±6.0	77.9±6.0
VP-4539, 1 mM	92.4±5.6	26.6±5.6	39.3±3.7	11.7±3.7
VP-4539, 2.5 mM	30.3±1.4	8.7±1.4	2.81±1.80	0.8±1.8

Note: [#]The comparison of fungi viability was performed toward the start control (C0) point and after 4 h of incubation (C4) with studied compound

3T3 cells, respectively. Doxorubicin was used as a reference positive control drug. Doxorubicin showed higher cytotoxic effect than studied compound **VP-4539** towards HaCaT and Balb/c 3T3 cells with the IC_{50} value of 0.8 and 0.9 μM, respectively.

Moreover, studied compound **VP-4539** demonstrated a low toxicity toward mitogen-activated lymphocytes isolated from healthy adult human peripheral blood. The IC_{50} value of **VP-4539** was 2.168 mM (Fig. 3). DMSO at the dose of 2.5 mM inhibited the growth of lymphocytes by 46.0%. The IC_{50} value of doxorubicin was 2.6 μM towards mitogen-activated lymphocytes isolated from healthy adult human peripheral blood.

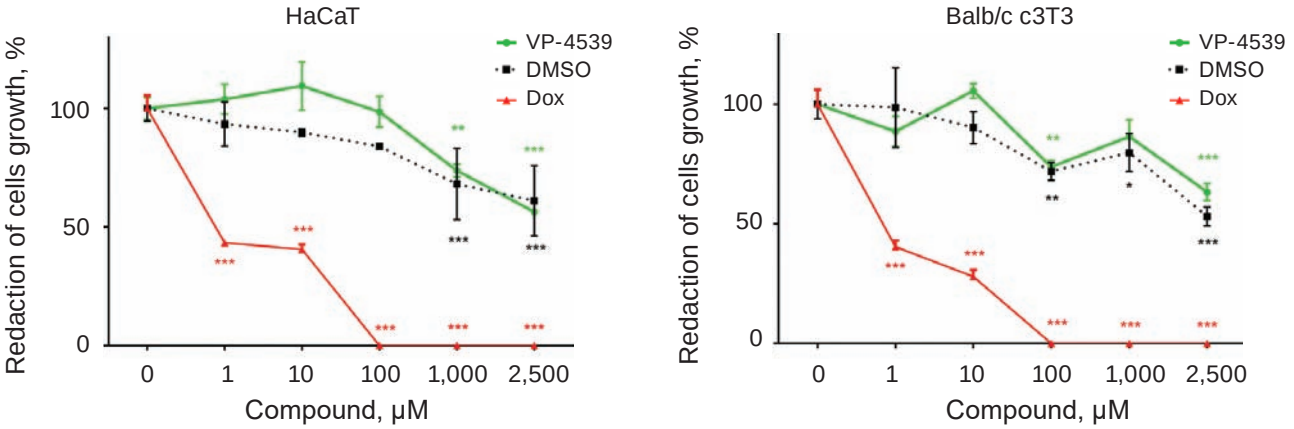


*Fig. 2. Cytotoxicity of studied compounds towards human keratinocytes of HaCaT line, and murine fibroblasts of Balb/c 3T3 line. After a total experimental time (72 h), cell vitality was evaluated by the MTT assay. Dox – doxorubicin. **P ≤ 0.01; ***P ≤ 0.001 (difference compared with the not treated control cells*

DNA intercalation study. It was reported that the several anticancer agents and other bioactive compounds can bind DNA and, thus, damage its structure resulting in the impairment of its function leading to cell death [33].

The compound **VP-4539** used at 1-100 μM concentrations insignificantly (0.7-5.7%) replaced the methyl green from the DNA-methyl green complex (Fig. 4). The ampicillin (negative control [34]) showed similar ability of methyl green replacement (0.8-4.0 %), while the doxorubicin (positive control and chemotherapeutic agent that intercalates into DNA structure [33]) replaced the methyl green by 15.8-27.6%. Ethydium bromide, that is classical intercalating agent, demonstrated 19.9-29.3% of methyl green replacement.

Based on the results of the methyl green replacement study, one may conclude that the compound **VP-4539** does not intercalate into DNA molecule and it is not the mechanism of its cytotoxic

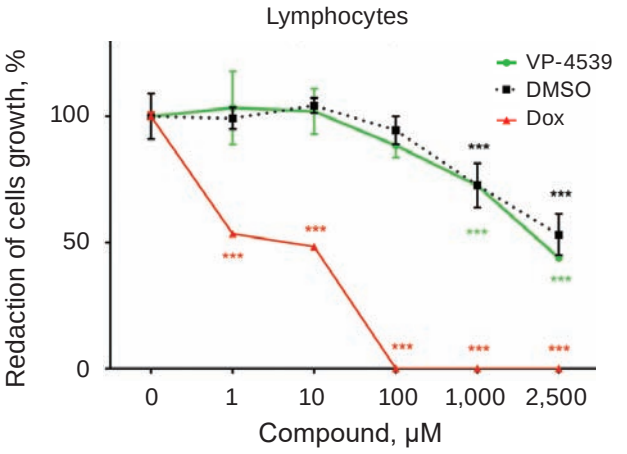


*Fig 3. Cytotoxicity of studied compound **VP-4539** towards mitogen-activated lymphocytes isolated from healthy adult human peripheral blood. After a total experimental time (48 h), cell vitality was evaluated by the MTT assay. Dox – doxorubicin. ***P ≤ 0.001 (difference compared with the not treated control cells)*

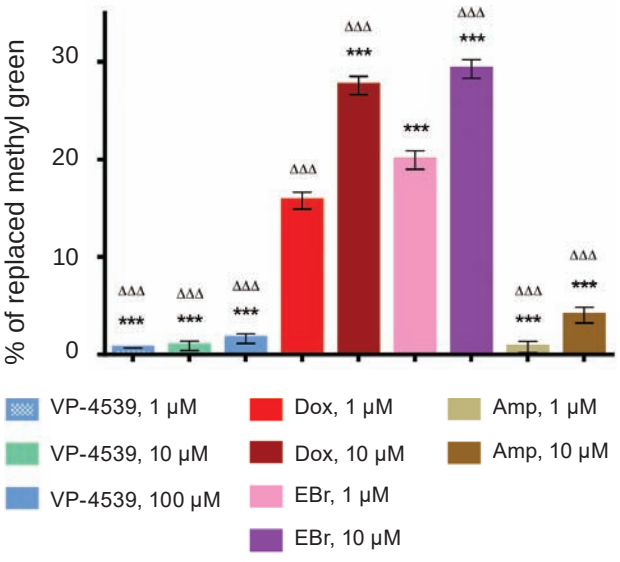


*Fig. 4. The results of a replacement of methyl green from DNA-methyl green complex by the compound **VP-4539**, doxorubicin (Dox), ethidium bromide (EtBr), and ampicillin (Amp). ***P < 0.001 (significant changes compared with Dox (1 μM) effect); ΔΔΔP < 0.001 (significant changes compared with EtBr (1 μM) effect)*

action. Other one may speculate that the DNA is not a primary target of compound **VP-4539**, other organelles surrounded by membranes may be such targets. Therefore, the damage of such structures could lead to the downstream effects, e.g. the ROS production, DNA damage. As the prokaryotes can live without a nucleus these cells could be more resistant to the action of studied compound. These assumptions need more dipper investigation.

Previously, it was reported that a series of dinuclear nickel(II) and copper(II) complexes of hexaazamacrocycles of 2,6-diformyl-4-methylphenol with three different benzoyl pendant-arms possessed the antibacterial activity and bound to calf thymus DNA by intercalative mode and cleavage the plasmid DNA [33]. The toxicity of metal-salen complexes based on the 1,2-bicyclo[2.2.2]octane bridge was investigated toward HCT116 colon carcinoma cells. The Mn(III)-salen complex possessed high toxicity for HCT116 cells. Milbeo et al. (2021) suggested that the compound containing the bicyclic structure could not intercalate into DNA molecule [16].

Other mechanisms of action of cage molecules were considered. It was reported that 2,2′-bis(bicyclo[2.2.1]heptane) under aerobic conditions caused the formation of the superoxide anion radical and hydrogen peroxide in *E. coli* cells that resulted in the damage of DNA [15]. The (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740) was reported as a selective agonist for group II metabotropic glutamate receptors (mGlu receptors 2 and 3) and potent agent for treatment of anxiety disorders [35, 36]. The bicyclo[2.2.1]heptane containing N,N′-diarylsquaramide was investigated as CXC chemokine receptor 2 antagonist. It demonstrated high cytotoxicity toward highly metastatic CFPAC1 pancreatic cancer cells, and inhibited their migration [37]. The 3-bicyclo[2.2.1]hept-2-yl-benzene-1,2-diol inhibited K^+ channels in mammalian cells with the EC_{50} values of 60 for Kir2.1 channels and 1 μM – for Kv2.1 channel. This compound abrogated *in vitro* apoptosis in neurons [38]. Manner et al. synthesized series of spirobicyclo[2.2.2]octane derivatives that possessed toxicity for MCF-7, SK-BR-3, CC1973, and L56Br-Cl a breast cancer cells with IC_{50} higher 22 μM. These derivatives did not stimulate microtubule polymerization in breast cancer cells. The IC_{50} higher 36 μM was reported for MCF-10A normal-like breast cells [39].

Thus, the application of bicyclic motifs of polycyclic scaffolds, such as bicyclo[2.2.1], bicyclo[2.2.2], bicyclo[4.4.1] and bicyclo[5.3.1] might be potent strategy in drug discovery.

Conclusions. In summary, we designed and synthesized the serial of cage amides and imides and tested for antimicrobial activity toward seven drug-resistant bacteria and fungal infections strains. It was found, that compound **VP-4539** with bicyclo[2.2.2]octene motif demonstrated promonnt growth inhibition activity towards *Cryptococcus neoformans* and was tolerant to human keratinocytes HaCaT line, murine fibroblasts Balb/c 3T3 line and mitogen-activated lymphocytes. Moreover, the presence of differences in the antifungal activity of this compound relative to laboratory and resistant strains indicates the potential of this substance as an element in the development of both synthesis techniques and perspective chemical constructs to overcome fungal multidrug-resistant infections. The obtained data will be used for further development and will be taken in the scaffold optimization. The compound **VP-4539** (1-100 μM) insignificantly (0.7-5.7%) replaced the methyl green from the DNA-methyl green complex.

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Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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СИНТЕЗ НОВИХ КАРКАСНИХ АМІДІВ ТА ІМІДІВ І ОЦІНКА ЇХ АНТИБАКТЕРІАЛЬНОЇ ТА ПРОТИГРИБКОВОЇ АКТИВНОСТІ

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Каркасні аміди та іміди, що містять біцикло[2.2.1]- та біцикло[2.2.2]-фрагменти синтезовано та оцінено на антимікробну активність щодо п'яти ключових патогенних ESKAPE бактерій: однієї грампозитивної бактерії метицилін-резисцентного *Staphylococcus aureus* (ATCC 43300), чотирьох грамнегативних бактерій *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606) і *Pseudomonas aeruginosa* (ATCC 27853). Оцінювали також сполуки на протигрибкову активність щодо патогенних штамів грибів *Candida albicans* (ATCC 90028) і *Cryformans necoccus var. Grubii* (H99; ATCC 208821). Сполука **VP-4539** з біцикло[2.2.2] октеновим фрагментом продемонструвала найвищу цитотоксичну активність щодо *C. neoformans* та, водночас, була толерантною до кератиноцитів людини лінії HaCaT, мишачих фібробластів лінії Balb/c 3T3 та мітоген-активованих лімфоцитів периферичної крові людини. Сполука **VP-4539** не вбудовувалася в ДНК сперми лосося, що свідчить про те, що її цитотоксичність не пов'язана з інтеркаляцією у ДНК.

Ключові слова: каркасні сполуки, біцикло[2.2.1]гептан, біцикло[2.2.2]октен, антимікробна та протигрибкова активність, цитотоксичність, інтеркаляція у ДНК, кератиноцити людини, лімфоцити.

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