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Investigation of Antimicrobial Activity of 1,3-benzoxazine Derivatives

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Aim. To investigate potential antimicrobial activity of 1,3-benzoxazines derivatives. **Methods.** Synthesis, antimicrobial screening, antimicrobial and antifungal activity testing *in vitro*. **Results.** For antimicrobial screening, we chose the compounds that differed from the investigated ones by 30 %. Five compounds were selected and their antimicrobial activity against five bacterium and two fungus strains was studied by the methods of microbial growth inhibition assay. The percentage of growth inhibition of an individual sample is calculated considering a negative control (media only) and a positive control (bacterial / fungal media without inhibitors). The results obtained showed that compounds 2-[5-(4-nitrophenyl)-1*H*-pyrazol-3-yl]phenol and 6,8-diisopropylspiro[1,3-benzoxazine-2,1'-cyclohexan]-4(3*H*)-one in concentration of 32 μg/ml have the highest activity against *Acinetobacter baumannii* - 43 % and 27 %, respectively. The compound, 6,8-diisopropyl-2-methyl-2-(4-nitrophenyl)-2,3-dihydro-4*H*-1,3-benzoxazine-4-one showed the fungicidal activity against the *Candida albicans* strain. **Conclusion.** The derivatives of 1,3-benzoxazines exhibit a moderate antimicrobial activity, which allows the recommendation to continue the search for effective antimicrobials among the chemical compounds of this group, in particular, through the targeted synthesis of new compounds with predicted antimicrobial properties.

Keywords: 1,3-benzoxazines, 1*H*-pyrazol-3-yl-phenol, antimicrobial screening

Introduction

Today, the problem of finding new effective medicines remains topical, despite the fact that branded medicines of various pharmacological groups are widely represented in the modern pharmaceutical market. One of the promising classes of chemical compounds, from the point of view of obtaining new biologically active substances on their basis, is the derivatives of benzoxazines. Professional journals quite often publish new information about the possibilities

of pharmacological application of the derivatives of compounds of this class. Benzoxazines are reported to be used as th potent progesterone receptor agonists, DNA-binding antitumor agents, human leukocyte elastase (HLE) and Clr serine protease inhibitors, as well as the fungicidal, anti-inflammatory and anticonvulsant drugs [1]. The derivatives of 1,4-benzoxazine exhibit the inhibitory activity in cell proliferation, which impedes the endothelial cell migration, and inhibition of angiogenesis in the chorioallantoic membrane assay [2]. Among the derivatives of 1,2-dihydrobenzoxazines, some promising compounds with biological activity have also been identified [3-5]. In particular, the study of 6-aryl-1,2-dihydro-4*H*-3,1-benzoxazine and 6-aryl-1,2-dihydro-4*H*-3,1-benzoxazine-2-thione has led to the development of potent and selective nonsteroidal progesterone-receptor agonists. Acridines with 1,2-dihydrobenzoxazines have demonstrated the cytotoxic activity against some lines of human cancer. The benzoxazine fragments are often found in natural compounds. For example, four alkaloids with antimicrobial action have been allocated from the sea sponge *Jaspis splendans*. One of them contains a substituent of the benzoxazine type [6]. High practical significance has prompted us to investigate the antimicrobial activity of 1,3-benzoxazines derivatives.

Materials and Methods

Methodology for the research] of the antibacterial activity

All bacteria were cultured in Cation-Adjusted Mueller-Hinton Broth (CAMHB) at 37 °C overnight. Each sample was diluted 40 times in a fresh medium and then, incubated at 37 °C for 1.5-3 hours. The samples of the mean logarithmic phase were diluted (4.5-5 × 10^5 CFU/ml, measured by 600 nm (OD600)). Then, the compounds containing the plates were added to each well, yielding a cell density of 5 × 10^5 CFU/ml and a total volume of 50 µl. All plates were coated and incubated at 37 °C for 18 hours without shaking.

Inhibition of growth of all bacteria was determined measuring absorbance at 600 nm (OD600), using a Tecan M1000 Pro monochromator plate reader. 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 reference.

Methodology of research of the antifungal activity

The fungi strain was cultivated for 3 days on YPD at 30 °C. A suspension of yeast from 1 × 10^6 to 5 × 10^6 CFU / ml (as defined by OD530) was prepared from five colonies. The suspension was then diluted and added to each well of the plates containing the compound, which gave the final density of fungi cells suspension of 2.5 × 10^3 CFU / ml and a total volume of 50 µl. All plates were coated and incubated at 35 °C for 36 hours without shaking.

The growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm (OD530). The growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm (OD600-570), after the addition of resazurin (0.001 % final concentration) and incubation at 35 °C for additional 2 hours. The absorbance

was measured using Biotek Synergy HTX Microplate Reader. The percentage of growth inhibition was calculated for each well, using negative control (media only) and positive control (fungi without inhibitors) on one plate.

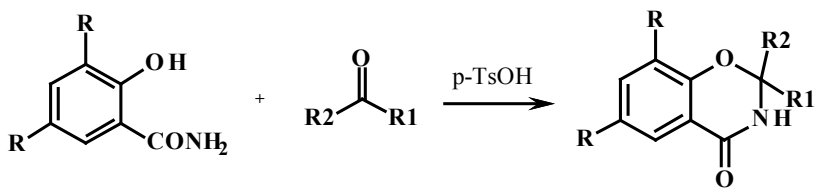
The percentage [of] growth inhibition of an individual sample is calculated based on negative controls (media only) and positive controls (bacterial / fungal media without inhibitors). The negative inhibition values indicate that the growth rate (or OD600) is higher compared to the negative control (bacteria / fungi only set to 0 % inhibition). The growth rate for all bacteria and fungi has a variation of — + 10 %, which is within the reported normal distribution of bacterial / fungal growth.

Results and Discussion

The methods of synthesis of geminal 1,3-benzoxazines derivatives had been developed in the previous studies authors. The Derivatives of 1,3-benzoxazines (1-4) have been obtained through the interaction of substituted salicylamides with ketones in benzene in the presence of p-TsOH removing water with a Dean-Stark trap (Scheme 1) [11, 12].

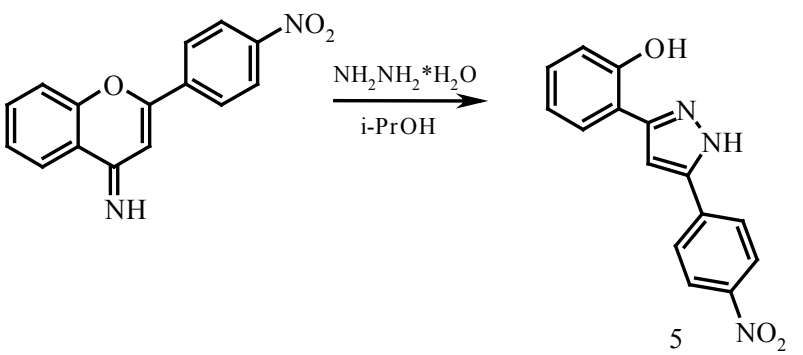
The 2-[5-(4-Nitrophenyl)-1*H*-pyrazol-3-yl] phenol (5) (Scheme 2) was obtained in the reaction of 2-(4-nitrophenyl)-4*H*-chromen-4-imine with hydrazine hydrate [12].

In cooperation with the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine, we sampled the synthesized compounds. These compounds were compared with the compounds whose antimicrobial activity had already been investigated (CHEMBL [13] and DrugBank [14] databases.) For antimicrobial screening, we chose the compounds that differed from the investigated ones by 30 %. Five compounds were selected and their antimicrobial activity against five bacterium and two



1 – R= i-Pr, R1=R2=$(CH_2)_4$; *2* – R=H, R1=CH_3, R2=4-NO_2-C_6H_4; *3* – R= i-Pr, R1= CH_3, R2= Ph; *4* – R= i-Pr, R1= CH_3, R2= 4-NO_2-C_6H_4;

Scheme 1. Synthesis of 1,3-benzoxazines derivatives



Scheme 2. Synthesis of 2-[5-(4-Nitrophenyl)-1*H*-pyrazol-3-yl]phenol 5

Table 1. **Microbial strains and cell lines.**

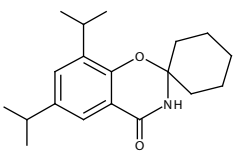
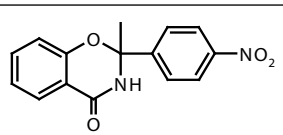
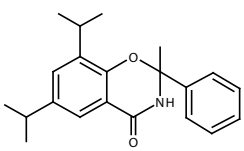
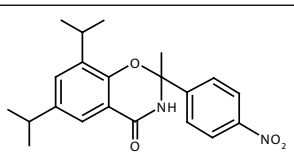
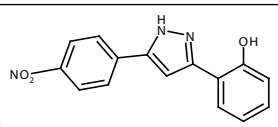
Abbreviation	Name	Description	Strain	Organsim	Type
Sa	*Staphylococcus aureus*	MRSA	ATCC 43300	Bacteria	G+ve
Ec	*Escherichia coli*	FDA control	ATCC 25922	Bacteria	G-ve
Kp	*Klebsiella pneumoniae*	MDR	ATCC 700603	Bacteria	G-ve
Ab	*Acinetobacter baumannii*	Type strain	ATCC 19606	Bacteria	G-ve
Pa	*Pseudomonas aeruginosa*	Type strain	ATCC 27853	Bacteria	G-ve
Ca	*Candida albicans*	CLSI reference	ATCC 90028	Fungi	Yeast
Cn	*Cryptococcus neoformans var. grubii*	H99 - Type strain	ATCC 208821	Fungi	Yeast

fungus strains was studied by the microbial growth inhibition assay.

The data on the tested strains of microorganisms are given in Table 1.

The screening is performed as two replica (n = 2), with both replicas on different assay plates, but from single plating and performed in a single screening experiment (microbial incubation). Each individual value is reported in the Tables (see 2 and 3).

Table 2. **The percentage of growth inhibition of whole cells by synthesized compounds**

Compound	*Strain*						
	(Sa)	*(Ec)*	*(Kp)*	*(Pa)*	*(Ab)*	*(Ca)*	*(Cn)*
1 	8.0	2.3	5.5	3.9	26.8	9.1	-9.1
2 	7.6	-4.0	1.8	1.3	8.5	11.3	-5.3
3 	16.2	3.7	5.8	0.9	3.0	5.6	-11.6
4 	7.5	0.3	2.2	-2.8	6.4	33.8	-3.6
5 	16.4	5.0	3.4	5.3	43.0	8.1	-57.4

During the analysis of the results obtained, it was found that among the synthesized compounds the most active with respect to *Acinetobacter baumannii* were the compounds (**5)** and (**1)** (Table 2 and Figure 1).

The repeated experiment confirmed the activity of compounds numbered (**5)** and (**1)** with regard to the *Acinetobacter baumannii* strain, as well as the activity of compound **(4)** in relation to the *Candida albicans* fungus (Table 3 and Figure 2).

Conclusions. According to the results of *in vitro* biological testing, it has been shown that the highest activity among the synthesized compounds

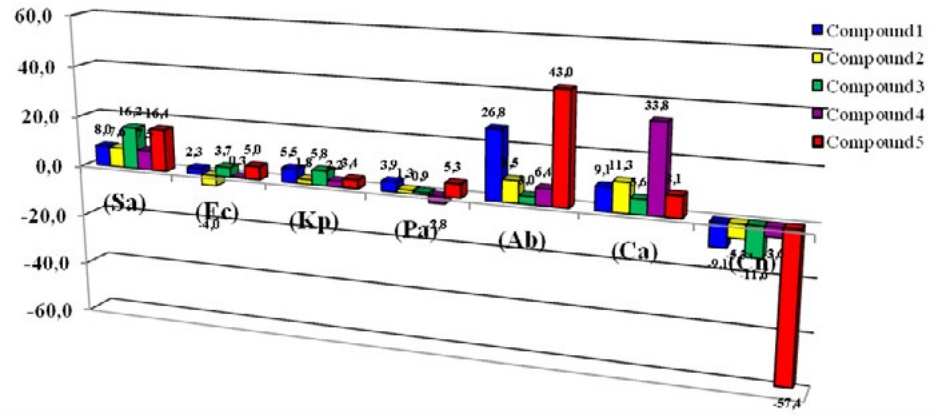


Fig. 1. Graph of growth inhibition of whole cells microorganisms by synthesized compounds (заменить запятые точками в графике)

with respect to *Acinetobacter baumannii* had the compounds 2-[5-(4-nitrophenyl)-1*H*-pyrazol-3-yl]phenol (**5**) and 6,8-diisopropylspiro[1,3-benzoxazine-2,1'-cyclohexan]-4(3*H*)-one (**1**), in concentration of 32 μg/ml (43 % and 27 %, respectively). Compound (**4**), 6,8-diisopropyl-2-methyl-2-(4-nitrophenyl)-2,3-dihydro-4*H*-1,3-benzoxazine-4-one, has shown the fungicidal activity against the *Candida albicans* strain.

Acknowledgements

The authors thank Prof. Sergii Yarmoluk from the Institute of Molecular Biology and Genetics NAS of Ukraine for the help in organization of antimicrobial screening.

Antimicrobial screening was conducted by CO-ADD (The Community for Antimicrobial Drug Discovery), Wellcome Trust (UK) and the University of Queensland (Australia).

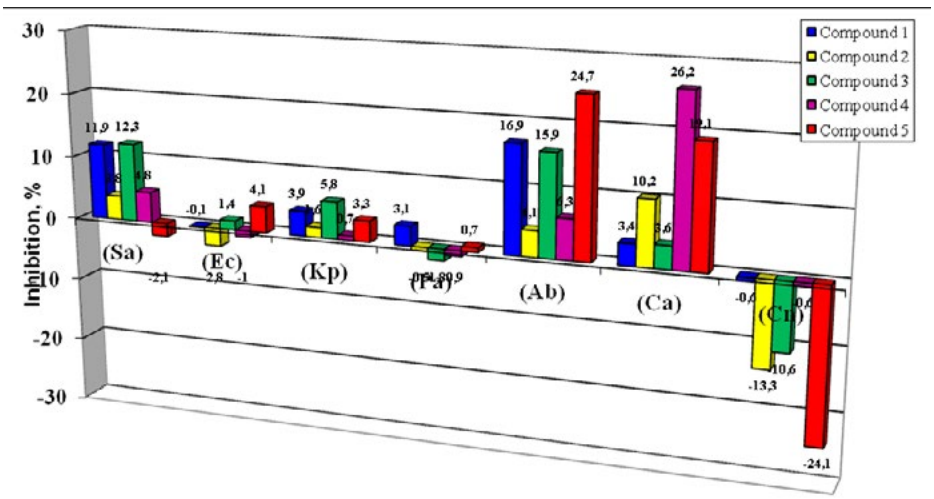
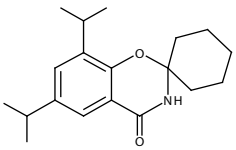
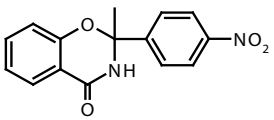
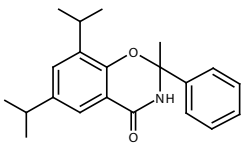
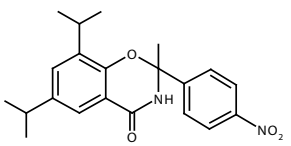
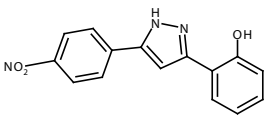


Fig. 2. Graph of growth inhibition of whole cells microorganisms by synthesized compounds (заменить запятые точками в графике)

Table 3. **The percentage of growth inhibition of whole cells by synthesized compounds**

Compound	*Strain*						
	(Sa)	*(Ec)*	*(Kp)*	*(Pa)*	*(Ab)*	*(Ca)*	*(Cn)*
1 	11,9	-0,1	3,9	3,1	16,9	3,4	-0,6
2 	3,8	-2,8	1,6	-0,5	4,1	10,2	-13,3
3 	12,3	1,4	5,8	-1,8	15,9	3,6	-10,6
4 	4,8	-1	0,7	-0,9	6,3	26,2	-0,6
5 	-2,1	4,1	3,3	0,7	24,7	19,1	-24,1

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Дослідження антимікробної активності похідних 1,3-бензоксазину

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Мета. Дослідити наявність антимікробної активності серед похідних 1,3-бензоксазинів. **Методи.** Синтез, антимікробний скринінг, біохімічне тестування *in vitro.* **Результати.** Для антимікробного скринінгу вибиралися сполуки які відрізняються від досліджених на 30 %. Було відібрано 5 сполук і проведено дослідження їх антимікробної активності проти п'яти бактерій та двох грибів методами аналізу інгібування росту цілих клітин. Інгібування росту індивідуального зразка розраховувався у відсотках на основі негативних контролів (лише середовищ) та позитивних контролів (бактеріальних / грибкових середовищ без інгібіторів). У ході аналізу отриманих результатів було встановлено що найбільшу активність серед синтезованих сполук проявили по відношенню до *Acinetobacter baumannii* сполуки - 2-[5-(4-нітрофеніл)-1*H*-піразол-3-іл]фенол та 6,8-діізопропілспіро[1,3-бензоксазін-2,1'-циклогексан]-4(3*H*)-он 43 % та 27 % при концентрації 32 мкг/мл. Фунгіцидну активність щодо штаму *Candida albicans* проявила сполука - 6,8-діізопропіл-2-метил-2-(4-нітрофеніл)-2,3-дигідро-4*H*-1,3-бензоксазин-4-он. **Висновки.** Похідні 1,3-бензоксазинів проявляють помірну антимікробну активність, що дозволяє рекомендувати продовження пошуку ефективних протимікробних засобів серед даного класу сполук, у тому числі і завдяки цілеспрямованому синтезу нових сполук з прогнозованими протимікробними властивостями.

Ключові слова: похідні 1,3-бензоксазинів, 1*H*-піразол-3-іл-фенол, антимікробний скринінг.

Исследование антимикробной активности производных 1,3-бензоксазинов

С. П. Загорулько, С. А. Вареніченко, О. К. Фарат, И. В. Маркова, В. И. Марков

Цель. Исследовать наличие антимикробной активности среди производных 1,3-бензоксазинов. **Методы.** Синтез, антимикробный скрининг, биохимическое тестирование *in vitro.* **Результаты.** Для антимикробного скрининга отбирали соединения, которые отличаются от изученных на 30 %. Было отобрано 5 соединений и проведено исследование их антимикробной активности против пяти бактерий и двух грибов методами анализа ингибирования роста целых клеток. Ингибирование роста индивидуального образца рассчитывается в процентах на основании отрицательных контролей (только среда) и положительных контролей (бактериальной / грибковой среды без ингибиторов). В ходе анализа полученных результатов было установлено что наибольшую активность среди синтезированных соединений проявили по отношению к *Acinetobacter baumannii соединения* - 2-[5-(4-нитрофенил)-1*H*-пиразол-3-ил] фенол и 6,8-диизопропилспиро[1,3-бензоксазин-2,1'-циклогексан]-4(3*H*)-он 43 % и 27 % при концентрации 32 мкг/мл. Фунгицидную активность против штамма *Candida albicans* проявило соединение - 6,8-диизопропил-2-метил-2-(4-нитрофенил)-2,3-дигидро-4*H*-1,3-бензоксазин-4-он. **Выводы.** Производные 1,3-бензоксазинов проявили умеренную антимикробную активность, что позволяет рекомендовать продолжение поиска эффективных противомикробных средств среди данного класса соединений, в том числе и благодаря целенаправленному синтезу новых соединений с прогнозируемыми противомикробными свойствами.

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