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Spiro[benzo[*e*]pyrano[3,2-*c*][1,2]oxathiin-4,3'-indolil]-3-carbonitrile 5,5-dioxides: synthesis and the biological activity study

The development of medicines with several pharmacological activities, including the analgesic, anti-inflammatory and antimicrobial properties, is one of the challenging tasks of modern medicinal chemistry.

Aim. To expand the range of novel spiro-condensed derivatives of 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide, and study the biological activity of the substances obtained.

Results and discussions. The target compounds were synthesized as a result of the interaction of 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide, malononitrile and isatins. When using ethyl cyanoacetate the interaction appeared to be much more complicated and requires further research. The study of the biological activity has revealed the compounds with the analgesic properties and the antimicrobial effect against gram-positive strains.

Experimental part. Two new 2-amino-2'-oxospiro[4*H*-pyrano[3,2-*c*][1,2]benzoxathiine-4,3'-indoline]-3-carbonitrile 5,5-dioxides were synthesized by the three-component reaction based on 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide. The anti-inflammatory activity was studied on the model of the carrageenan induced paw edema, and the analgesic activity was assessed on the model of the local inflammatory hyperalgesia. The study of the antimicrobial activity of the compounds obtained was performed by the agar well diffusion method.

Conclusions. New spiro[benzo[*e*]pyrano[3,2-*c*][1,2]oxathiin-4,3'-indolil]-3-carbonitrile 5,5-dioxides have been synthesized. The compounds obtained have revealed high levels of the analgesic properties and the antimicrobial activity. The latter exceeds the activity of the reference drugs, and has appeared to be higher against gram-positive bacteria.

Key words: 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide; isatins; spiro compounds; analgesic activity; anti-inflammatory activity; antimicrobial activity

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Спіро[бензо[*e*]пірано[3,2-*c*][1,2]оксатіїн-4,3'-індол]-3-карбонітрил 5,5-діоксиди: синтез і вивчення біологічної активності

Розробка лікарських засобів, що володіють декількома видами фармакологічної активності, включаючи знеболювальну, протизапальну та антимікробну, є одним з важливих завдань сучасної медичної хімії.

Мета. Розширити ряд нових спіроконденсованих похідних 1,2-бензоксатіїн-4(3*H*)-он 2,2-діоксиду і дослідити біологічну активність одержаних речовин.

Результати та їх обговорення. Цільові сполуки були синтезовані в результаті взаємодії 1,2-бензоксатіїн-4(3*H*)-он 2,2-діоксиду, малонодинітрилу та ізатинів. У випадку використання етилціаноацетату як метиленактивного нітрилу взаємодія виявилася набагато складнішою і потребує подальших досліджень. Вивчення біологічної активності виявило сполуки з анальгетичними властивостями та антимікробною дією проти грампозитивних штамів.

Експериментальна частина. Два нових 2-аміно-2'-оксоспіро[4*H*-пірано[3,2-*c*][1,2]бензоксатіїн-4,3'-індолін]-3-карбонітрил 5,5-діоксиди були синтезовані за допомогою трикомпонентної реакції на основі 1,2-бензоксатіїн-4(3*H*)-он 2,2-діоксиду. Протизапальну активність вивчали на моделі карагенінового набряку, анальгетичну активність оцінювали на моделі місцевої запальної гіпералгезії. Були проведені дослідження антимікробної активності отриманих сполук методом дифузії в агар.

Висновки. Синтезовано нові спіро[бензо[*e*]пірано[3,2-*c*][1,2]оксатіїн-4,3'-індол]-3-карбонітрил 5,5-діоксиди. Отримані сполуки виявили високий рівень анальгетичної та антимікробної активності. Остання перевищує активність референс-препаратів і виявилася більш ефективною проти грампозитивних бактерій.

Ключові слова: 1,2-бензоксатіїн-4(3*H*)-он 2,2-діоксид; ізатини; спіроконденсовані сполуки; анальгетична активність; протизапальна активність; антимікробна активність

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Спиро[бензо[*e*]пирано[3,2-*c*][1,2]оксатиин-4,3'-индол]-3-карбонитрил 5,5-диоксиды: синтез и изучение биологической активности

Разработка лекарственных средств с несколькими видами фармакологической активности, включая анальгетические, противовоспалительные и антимикробные свойства, является одной из важных задач современной медицинской химии.

Цель. Расширить ряд новых спироконденсированных производных 1,2-бензоксатиин-4(3*H*)-он 2,2-диоксида и изучить биологическую активность полученных веществ.

Результаты и их обсуждение. Целевые соединения были синтезированы в результате взаимодействия 1,2-бензоксатиин-4(3*H*)-он 2,2-диоксида, малонодинитрила и изатинов. В случае использования этилцианоацетата в качестве метиленактивного нитрила взаимодействие оказалось значительно более сложным и нуждается в дальнейшем изучении. Исследование биологической активности выявило соединения с анальгетическими свойствами и антимикробным действием в отношении грамположительных штаммов.

Экспериментальная часть. Два новых 2-амино-2'-оксоспиро[4*H*-пирано[3,2-*c*][1,2]бензоксатиин-4,3'-индолин]-3-карбонитрил 5,5-диоксида были синтезированы с помощью трехкомпонентной реакции на основе 1,2-бензоксатиин-4(3*H*)-он 2,2-диоксида. Противовоспалительную активность изучали на модели карагенин-индуцированного отека, а анальгетическую активность оценивали на модели локальной воспалительной гипералгезии. Было проведено исследование антимикробной активности полученных соединений методом диффузии в агар.

Выводы. Синтезированы новые спиро[бензо[*e*]пирано[3,2-*c*][1,2]оксатиин-4,3'-индолил]-3-карбонитрил 5,5-диоксиды. Полученные соединения проявили анальгетические свойства и антимикробную активность, которая превышает активность препаратов сравнения и оказалась выше в отношении грамположительных бактерий.

Ключевые слова: 1,2-бензоксатиин-4(3*H*)-он 2,2-диоксид; изатины; спироконденсированные соединения; анальгетическая активность; противовоспалительная активность; антимикробная активность

To date the search of novel medicines with the anti-inflammatory and analgesic properties still remains one of the challenging tasks in medicinal chemistry [1, 2]. The reason is that pain belongs to the most unpleasant feelings among other symptoms. It can be an independent problem, for example, in the case of migraine, or it can accompany injuries and surgical interventions, or can be one of the symptoms of the musculoskeletal system chronic diseases [3]. However, in all cases, pain becomes a source of suffering and significantly disturbs the natural rhythm of life. It is important to note that chronic pain, regardless of the cause for its occurrence, is a threat to the patient's life since it causes such pathological conditions as depression, anxiety, sleep and immune disorders, constant tension of the cardiovascular system. Therefore, attempts to "endure" pain, as well as the recommendation "to learn how to live with pain", seems to be vicious and harmful [4].

In current therapeutic practice three groups of analgesics are used most widely. The first of them, essentially, consists of only one drug – acetaminophen (paracetamol, "simple analgesic"). The second type is represented by a very large group of non-steroidal anti-inflammatory drugs (NSAIDs): aceclofenac, diclofenac, ibuprofen, ketorolac, lornoxicam, meloxicam, metamizol, naproxen, nimesulide, piroxicam, tenoxicam, phenylbutazone, flurbiprofen, celecoxib, etoricoxib, etc. The third group is opioid analgesics that mostly belong to prescription drugs [5]. Unquestionably, this does not exhaust the entire arsenal of painkillers. There are specific drugs that are used to treat certain types of chronic pain. Furthermore, medicines with several pharmacological activities, including the analgesic one, are being also developed.

In our previous studies spiro-condensed derivatives of 1*H*-2,1-benzothiazin 2,2-dioxide **A** (Fig. 1) were proven to be a novel and promising class of compounds for treating disorders accompanied by inflammation and pain [6]. The antifungal activity was also revealed for compounds from this group. In continuation of these studies we paid our attention to 1,2-benzoxathiin 2,2-dioxide **B** derivatives as isosteric to the abovementioned compounds **A**.

As the first logical step a wide range of type **B** compounds **4a–n** was synthesized based on the three-component interaction of 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide, malononitrile and substituted isatins [7]. In the current study the range of 2-amino-4*H*-pyran-3-carbonitriles **4** was additionally expanded using

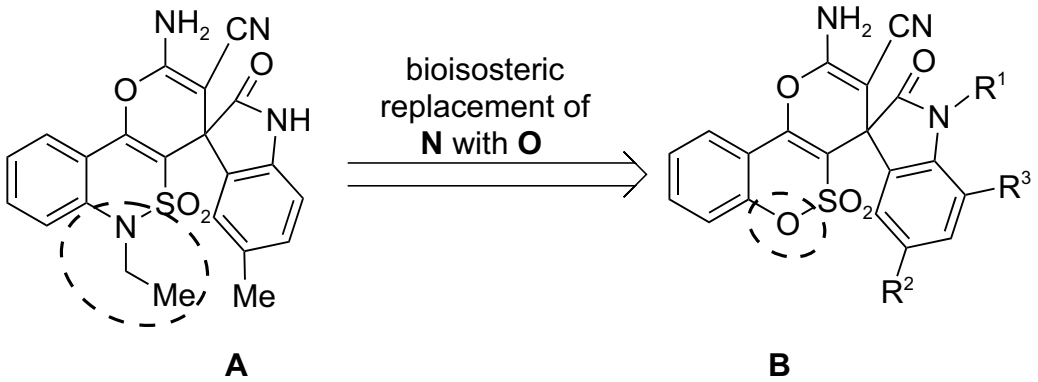
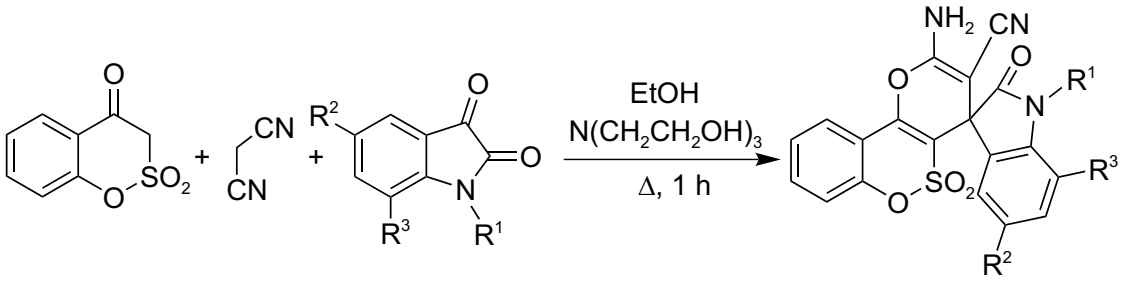


Fig. 1. Isosteric relationships between 1*H*2,1benzothiazin 2,2dioxide **A** and 1,2benzoxathiin 2,2dioxide **B** core

isatins **3o**,**p** (Scheme 1). There was also an attempt to use isatins **3q–s** in the same reaction, but no comprehensible results were obtained (because of tarring).

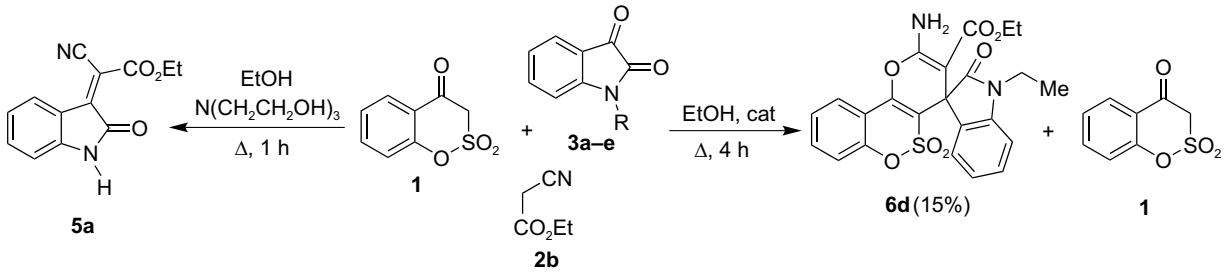
In order to extend the variety of spiro-condensed derivatives of 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide we replaced malononitrile with ethyl cyanoacetate **2b**. In the case of its use together with other carbonyls the complicated results were mostly obtained [7–9]. This time, carrying out the interaction under the same reaction conditions as for malononitrile **2a** gave ylidene **5a**, and the complex mixtures of the starting compounds with the ylidenes **5** and the target products **6** for isatins **3b,c**,**e** (Scheme 2). Previously, the use of *N*-ethylisatin allowed us to isolate the target spiro-derivative **6d** in a poor yield 5% [7]. Application of other basic catalysts in this research (triethylamine, DBU, sodium acetate) instead of triethanolamine led to a slight increase of **6d** yield (15%), but it was contaminated with 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide **1.** Thereby, the three-component interaction of 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide **1**, isatins **3** and ethyl cyanoacetate **2b** requires further detailed study. It is worth mentioning that application of the two-stage process, namely the synthesis



1 **2a** **3a–s** **4a–p**

№	R^1	R^2	R^3	Yield, %
4a	H	H	H	71
4b	CH_2COOEt	H	H	45
4c	Allyl	H	H	58
4d	Et	H	H	65
4e	Bn	H	H	85
4f	Me	H	H	91
4g	H	Me	H	95
4h	H	NO_2	H	84
4i	H	Br	H	88
4j	Allyl	Br	H	92
4k	Bn	Br	H	51
4l	Me	Br	H	91
4m	Me	Me	H	93
4n	H	Br	Br	36
4o	CH_2CONH_2	H	H	35
4p	$CH_2CONHPh$	H	H	33
4q	CH_2OH	H	H	–
4r	CH_2-(piperidin-1-yl)	H	H	–
4s	CH_2-(morpholin-4-yl)	H	H	–

Scheme 1. The interaction of 1,2benzoxathiin4(3*H*)one 2,2dioxide **1**, malononitrile **2a** and isatins **3**



Scheme 2. The interaction of 1,2benzoxathiin4(3*H*)one 2,2dioxide **1**, ethyl cyanoacetate **2b** and isatins **3**

of ylidene **5a** and its further reaction with 1,2-benzoxathiin-4(3*H*)-one 2,2dioxide **1**, resulted only in isolation of the initial compounds (Scheme 2).

According to the isosteric relationships between benzothiazine and benzoxathiine cores mentioned the following types of the biological activity was studied: analgesic, anti-inflammatory, antimicrobial.

The results of testing the analgesic and anti-inflammatory activities are given in Tab. 1. Compounds **4g, 4i** and **4m** had an average effect in the applied model of inflammation and decreased the edema development compared to the control group, but their activity was twice less than for the reference drug Piroxicam.

The analgesic activity of the compounds studied appeared to be generally higher, but variable. The ability to reduce the pain threshold at the level of Piroxicam was found for spiro[(2-amino-3-cyano-4,6-dihydropyrano[3,2-*c*][2,1]benzoxathiin-5,5-dioxide)-4,3'-(5-methylindolin-2'-one)] **4g**, but with high variability.

It should be noted that, in general, spiro-derivatives of benzoxathiine **4** displayed the lower level of the analgesic and anti-inflammatory activities compared to benzothiazine ones mentioned above.

The antimicrobial activity was assessed according to the international standards [10, 11] by the agar well diffusion method against the standard test-strains of gram-positive and gram-negative microorganisms and fungi. As reference drugs the common representatives of antibacterial (Synthomycin) and antifungal (Metronidazole*)* compounds were used. The results appeared to be higher than for the reference drugs. The moderate activity found was higher against gram-positive bacteria than against gram-negative bacteria and fungi in contrast to the isosteric derivatives of 1*H*-2,1-benzothiazin-4(3*H*)-one 2,2-dioxide, which antimicrobial properties were associated predominantly with the inhibitory effect on gram-negative strains and fungi. There was no significant distinction for N- and 5-substituted derivatives at the isatin core. The results of studying the antimicrobial properties are presented in Tab. 2.

Spiro[(2-amino-3-cyano-4,6-dihydropyrano [3,2-*c*][2,1]benzoxathiin-5,5-dioxide)-4,3'-(N-ethylindoline-2'-one)] **4d** seemed to be the most active antimicrobial agent among the compounds studied. Thus, the values of the minimum inhibitory concentration (32.25 μg/mL) and the minimum bactericidal concentration (62.5 μg/mL) were additionally found for it.

Experimental chemical part

The starting isatin and active methylene nitriles were obtained from commercial sources and used without further purification. 1,2-Benzoxathiin-4(3*H*)-one 2,2-dioxide **1** and isatins **3b-s** were prepared according to the procedures described in [7]. Melting points were determined on a Gallenkamp melting point apparatus, Model MFB-595 in open capillary tubes. ^{1}H NMR-spectra were recorded on a Varian WXR-400 spectrometer using DMSO-d_6 as a solvent and TMS as an internal standard. Elemental analyses were carried out using a Carlo Erba CHNS-O EA 1108 analyzer.

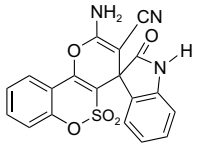
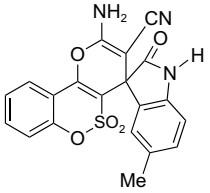
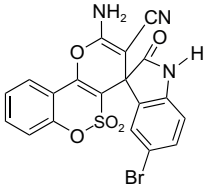
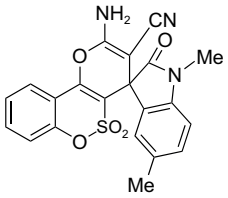
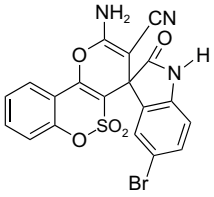
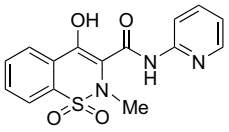
The general procedure for the synthesis of 2-amino-2'-oxo-1'-R-spiro[benzo[*e*]pyrano[3,2-*c*]-[1,2]oxathiin-4,3'-indolil]-3-carbonitrile 5,5-dioxides 4o,p. To the solution of 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide 1 (0.198 g, 0.001 mole), malononitrile **2a** (0.066 g, 0.001 mole) and the appropriate isatin **3o,p** (0.001 mole) in ethanol (5–10 mL) add 20 mole% of triethanolamine. Reflux the mixture for 1 h. Filter the precipitates of **4o,p** obtained, wash with ethanol and then dry in air.

2-(2-Amino-3-cyano-5,5-dioxido-2'-oxospiro-[benzo[*e*]pyrano[3,2-*c*][1,2]oxathiin-4,3'-indolin]-1'-yl)acetamide 4o. A brown crystalline powder. M. p. 185–187°C (EtOH); Anal. Calcd. for $C_{21}H_{14}N_4O_6S$, %: C 56.00, H 3.13, N 12.44. Found, %: C 55.87, H 2.95, N 12.61. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 7.90–7.98 (1H, m, Ar-H), 7.82 (2H, s, NH_2), 7.73–7.76 (1H, m, Ar-H), 7.57–7.63 (1H, m, Ar-H), 7.49–7.56 (1H, m, Ar-H), 7.41–7.48 (1H, m, Ar-H), 7.32–7.40 (2H, m, $CONH_2$), 6.96–7.16 (3H, m, Ar-H), 4.16–4.36 (2H, m, CH_2).

2-(2-Amino-3-cyano-5,5-dioxido-2'-oxospiro-[benzo[*e*]pyrano[3,2-*c*][1,2]oxathiin-4,3'-indolin]-1'-yl)-N-phenylacetamide (4p). A brown crystalline powder. M. p. 238–240°C (EtOH); Anal. Calcd. for $C_{27}H_{18}N_4O_6S$, %: C 61.59, H 3.45, N 10.64. Found, %: C 61.35, H 3.27, N 10.51. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 10.14 (1H, s, NH); 7.93–7.98 (1H, m, Ar-H); 7.80 (2H, s, NH_2); 7.71–7.77 (1H, m, Ar-H); 7.51–7.63 (4H, m, Ar-H); 7.44–7.49 (1H, m, Ar-H); 7.27–7.33 (2H, m, Ar-H); 7.01–7.14 (3H, m, Ar-H); 4.46–4.65 (2H, m, CH_2).

Table 1

The results of the study of the anti-inflammatory and analgesic activities of compounds **4**

Compound	Dose (mg/kg)	The average percentage of edema, %*	Anti-inflammatory activity, %*	Reducing the pain threshold, %*	Analgesic activity, %*
 **4a**	2.4	54.27 ± 3.93**	3.3 ± 2.2**	30.6 ± 9.0	46.8 ± 13.3
 **4g**	2.4	35.81 ± 1.65*,**	24.0 ± 3.5**	23.5 ± 5.8*	53.4 ± 11.5
 **4i**	2.8	36.25 ± 4.45**	24.6 ± 8.6**	38.3 ± 5.1**	27.7 ± 7.5**
 **4m**	2.5	42.02 ± 7.52**	23.5 ± 11.5	26.1 ± 6.5*	48.2 ± 13.0
 **4n**	3.3	51.61 ± 6.7**	8.0 ± 3.1**	29.3 ± 11.7	46.8 ± 21.0
 **Piroxicam**	2	23.81 ± 2.73*	49.4 ± 5.8	22.7 ± 3.3*	55.0 ± 6.6
Control	–	47.1 ± 3.2	–	50.4 ± 3.1	–

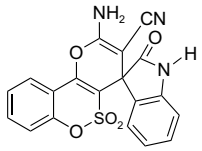
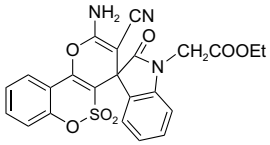
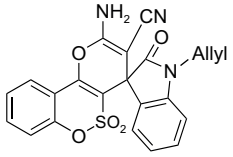
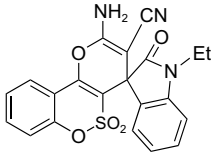
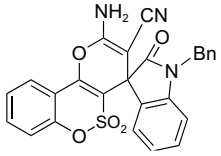
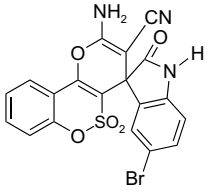
Notes: * – the deviation is valid for the control ($p \leq 0.05$); ** – the deviation is valid for Piroxicam ($p \leq 0.05$).

Experimental biological part

The study of the anti-inflammatory and analgesic activities was performed in albino adult male and female rats weighing 150–180 g. The animals were randomly divided into seven groups of equal number (control, 5 experimental and comparison groups). The use of Piroxicam (Chervona Zirka, Ukraine) as the reference drug was due to its isosteric relationships with the core of 1,2-benzoxathiine 2,2-dioxide. The anti-inflammatory activity was studied on the model of the carrageenan induced paw edema, and the analgesic activity was evaluated on the model of the local inflammatory hyperalgesia. Pathology in both cases was reproduced by the intraplantar injection of 0.1 mL of 1% solution of γ-carrageenan (Sigma, USA)

Table 2

The antimicrobial activity of spiro[benzo[*e*]pyrano[3,2-*c*][1,2]oxatiamine-4,3′-indolyl] 5,5-dioxides

Compound	Diameter of the growth inhibition zones (average for three experiments), mm					
	Gram-positive bacteria		Gram-negative bacteria		Fungi	
	S. aureus	*E. coli*	*B. subtilis*	*P. aeruginosa*	*P. vulgaris*	*C. albicans*
Metronidazole	14	14	16	0	0	14
Synthomycin	14	17	17	17	17	0
 **4a**	22	19	22	17	16	18
 **4b**	21	19	22	16	16	17
 **4c**	21	19	23	16	17	18
 **4d**	22	20	23	17	17	18
 **4e**	22	19	22	16	17	18
 **4i**	21	20	22	15	16	18

into the right hind limb of rats [12, 13]. The test compounds and the reference drug were introduced orally as fine aqueous suspensions stabilized with Tween-80 one hour prior to the γ-carrageenan injection. The screening dose for Piroxicam was 2 mg/kg, the test compounds were introduced in the doses that were equimolar to the reference drug. The control group received an equivalent amount of Tween-80 water solution.

The initial and final values of the paw edema volume were measured by the water displacement method using a digital plethysmometer (IITC Life Science, USA). The final values of the paw edema volume were received 3 h after the phlogogen agent injection.

The initial values of the pain threshold were measured using an Ugo Basile 37215 analgesimeter [14, 15], and the final values of the pain threshold were ob-

tained on the inflamed paw 2 h after administration of the test substances.

The anti-inflammatory activity (%) was expressed as percentage of edema inhibition in the animals treated with the test compound and Piroxicam compared to the control rats. The anti-inflammatory activity was calculated by the formula:

$$\text{AIA} = \frac{\Delta V_c - \Delta V_e}{\Delta V_c} \cdot 100\ \%,$$

where: AIA – is the anti-inflammatory activity, %; ΔV_c – is the average percentage of edema in the control group, %; ΔV_e – is the average percentage of edema in the experimental group (comparison group), %.

The analgesic activity was evaluated by the change of the pain threshold checked on the inflamed paw in the rats received the test compound and the reference drug compared to the animals from the control group. The analgesic activity was calculated by the formula:

$$\text{AA} = \frac{\Delta PT_c - \Delta PT_e}{\Delta PT_c} \cdot 100\ \%,$$

where: AA – is the analgesic activity, %; ΔPT_c – is the average percentage of the pain threshold decrease in the control group, %; ΔPT_e – is the average percentage of the pain threshold decrease in the experimental group (comparison group), %.

The results of biological tests were also processed by the method of variation statistics using Student's *t*-criterion and Mann–Whitney U Test, and such programs as STATISTICA 7.0, Stat-Plus 2009 and MS Excel 2007 [16–18]. The current study was carried out in full compliance with the Directive 86/609/EU of the European Parliament and of the Council of 24 November 1986 on protection of animals used for scientific purposes [19, 20].

Experimental microbiological part

According to the WHO recommendations [10], the following test-strains were used: *Staphylococcus aureus ATCC 25923*, *Escherichia coli ATCC 25922*, *Pseudomonas aeruginosa ATCC 27853*, *Bacillus subtilis ATCC 6633*, *Proteus vulgaris ATCC 4636*, *Candida albicans ATCC 653/885*. The inoculum suspension was prepared using a Densi-La-Meter apparatus (made by PLIVA-Lachema, Czech Republic; the wavelength of 540 nm).

The suspension was prepared according to the manual for the device and the information sheet No 163-2006 "Standardization for preparation of microbial suspensions" (Kyiv) about innovations in the healthcare system. The inoculum density was 10^7 cells in 1 mL of the medium, and it was determined by comparing to McFarland standard [21]. The 18 to 24-hour old cultures of the microorganisms were employed for the test. For the antimicrobial evaluation the Mueller–Hinton agar was used, for *Candida albicans* strain the Sabouraud agar was taken. The compounds were introduced into agar by the "well" method [10]. The antibacterial activity was evaluated by measuring zones of inhibition of the corresponding microorganism and was compared to those for the reference antimicrobial drugs.

The bacteriostatic and bactericidal activity of new compounds was determined in accordance with the requirements of the State Pharmacopoeia of Ukraine (ed. 1) by the double serial dilution method in the liquid growth medium. Testing was made in the volume of 1 mL of each dilution of substances with the final concentration of the microorganism under study about 5×10^5 CFU/mL. After incubation for a day or 48–72 hours for *Candida* spp. the cuvettes were seen in bright light to determine the presence of the microorganism growth. The minimum inhibitory concentration (MIC) was set at the lowest concentration of the test substance, which suppressed the apparent growth of the culture. To determine the minimum bactericidal concentration (MBC), dosage seeds were fed on a solid nutrient medium (Muller–Hillton agar) of the culture fluid from all the tubes, in which no growth of the microorganism was observed. The lowest concentration, which caused the death of at least 90% of bacteria, was taken as the MBC. The additional control was performed for the culture in the medium without the substances studied, in the solvent; the purity of the suspension with the microorganism (by seeding on the non-selective media) and the sterility of the medium were controlled.

Conclusions

1. The use of malononitrile in the three-component interaction with 1,2-benzoxathiin-4(3*H*)-one 2,2-dioxide and isatins has led to the formation of the target amino-2'-oxospiro[4*H*-pyrano[3,2-*c*][1,2] benzoxathiine-4,3'-indoline]-3-carbonitrile 5,5-dioxides. The replacement of malononitrile with ethyl cyanoacetate in the reaction has resulted in mixtures of the target and starting compounds.

2. The analgesic activity of the spiro-derivatives synthesized has appeared to be generally higher than the anti-inflammatory one.

3. The current study implies that 1,2-benzoxathiin 2,2-dioxide pyran-annulated derivatives are lower active than those constructed on the basis of 1*H*-2,1-benzothiazin-4(3*H*)-one 2,2-dioxide.

4. The compounds synthesized have appeared to be unpromising antimicrobial agents.

Conflict of interests: authors have no conflict of interests to declare.

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