PROMISING MATERIALS BASED ON TETRAHYDRO-[1,2,4]OXADIAZOLE[3,2-C][1,4]OXAZINE FOR INNOVATIVE BIOTECHNOLOGIES

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ABSTRACT
The synthesis of 2-[(1,3-diphenyl-1H-1,2,4-triazol-5-yl) dinitromethyl]-5,6,8,8a-tetrahydro-[1,2,4] oxadiazol[3,2-c] [1,4] oxazine is caused by the reaction of morpholine interaction in the presence of hydrogen peroxide with 2-(1,3-diphenyl-1H-1,2,4-triazol-5-yl)-2,2- dinitroacetonitrile and sodium tungstate in a catalytic amount. The latter interacts with an excess of KOH in ethanol to form the potassium salt aci-5-dinitromethyl-1,3-diphenyl-1H-1,2,4-triazole and 5,6,8,8a-tetrahydro-[1,2,4] oxadiazole[3,2-c] [1,4] oxazine-2-ol. The ability of compounds 3-5 to inhibit the growth and development of museum strains of Staphylococcus aureus RP, E. coli O39, Pseudomonas aeruginosa 143 and clinical strain of Streptococcus pneumonia, as well as clinically significant species of fungi Trichophyton rubrum, Microsporumcanis and Candida albicans was investigated. It was revealed that the compounds have bacteriostatic activity against the studied bacterial strains: **3** and **4** expressed in a range of low concentrations. Compounds **3-5** demonstrate a fungistatic effect: compound **3** in a range of low concentrations. In relation to fungal strains, compounds **3-5** demonstrate a fungistatic effect: compound **3** in the range of low concentrations. Thus, as a result of the implementation of the "one pot" process, it is possible to embed oxadiazoline and oxazine cycles into the main (central) part of the compound 1 molecule, which leads to the synthesis of new drugs with antimicrobial and antifungal activity.

Keywords: *2-(1,3-diphenyl-1H-1,2,4-triazol-5-yl)-2,2-dinitroacetonitrile, morpholine N-oxide, 1,3-dipolar cycloaddition reaction, hydrogen peroxide, sodium tungstate , 2-[(1,3-diphenyl-1H-1,2,4-triazol-5-yl)dinitromethyl]-5,6,8,8a – tetrahydro-[1,2,4]oxadiazole[3,2 -c][1,4]oxazine, aci-5-dinitromethyl-1,3-diphenyl-1H-1,2,4-triazole potassium salt, 5,6,8,8a-tetrahydro-[1,2,4]oxadiazol[3,2-c][1,4]oxazin-2-ol, antimicrobial activity, fungistatic activity.*

INTRODUCTION

The resistance of microorganisms of various types, including conditionally pathogenic ones, to antimicrobial drugs is an urgent problem that needs to be solved, since it does not allow for the full implementation of effective therapeutic measures.

Gram-negative pathogens - Escherichia coli and Pseudomonas aeruginosa (*E. coli* and *P. aegidiposa*) occupy a leading position in the implementation of in-hospital infections. Methicillin -resistant *Staphylococcus aureus* also occupies a significant place, which is an etiological cause of the development of infections both in hospitals and outside them. Microorganisms belonging to the genus *Streptococcus* are the cause of a diverse range of infectious diseases (including severe invasive ones), which is also a significant problem for healthcare [1, 2].

Trichophyton rubrum, having a high enzymatic activity, causes damage to the feet, skin, downy and long hair. Rubromycosis accounts for 60-80% of all cases of fungal foot diseases.

Microsporumcanis is the most frequently registered causative agent of microsporia in Russia. It refers to zoophilic fungi that are ubiquitous in the world, causing dermatophytosis.

Candida albicans is the causative agent of candidiasis. From 85 to 90% of yeast strains isolated from the vagina belong to *Candida albicans*. Currently, there is an increase in the rates of *Candida fungi* resistance to drugs widely used in therapy [3.

In this regard, the creation and development of new substances with antibacterial and antifungal activity is currently an actual task [4-7].

EXPERIMENTAL CHEMICAL PART

Materials and methods

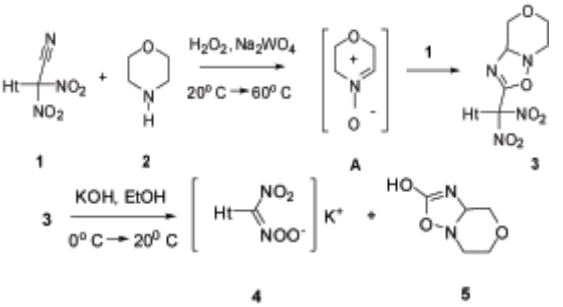
2-(1,3-Diphenyl-1H-1,2,4-triazol-5-yl)-2,2-dinitroacetonitrile 1 was obtained by the method [10], "reagents (sodium tungstate and hydrogen peroxide) of the "purissimum" brand of the company "ALDRICH". The IR spectrum of compounds 3 and 5 was taken in the frequency range 4000-400 cm-1 in chloroform on an InfraLUMFT-02 spectrophotometer (Russia). The NMR1H and 13C spectra were recorded with an operating frequency of 500 and 125 MHz, respectively, in DMSO-d_6, the internal standard is GMDS on the Bruker Avence II 300 SF device (Germany). The mass spectroscopic study was carried out in the direct input mode, the ionization energy is 70 eV, the evaporation temperature of the sample is 500-550 ° C on the Finnigan SSQ-7000 device (USA). " According to [13], "by the method of ascending TLC in the acetone-hexane 2:3 solvent system, iodine vapors on Silufol UV-254 plates were the developer, the course of the action and the individuality of the resulting compound were controlled." The elemental analysis was performed on an automatic Euro EA-3000 CHNS analyzer from Euro Vector (Italy), melting or decomposition temperature.

Results and their discussions

To get a new derivative pirimidin(2-arilltetrahydro-1′H,2H-spiro[ixazol[3,2-c][1,4]oxazin-3,5′-pirimidin]-2′6,′4,′('3H)-trions) the reaction of the interaction of 5-(arylmethylidene)-2,4,6-pyrimidine-2,4,6(1H,3H,5H)-triones with morpholine N-oxide was studied [8]. To study the synthetic possibilities of the reaction of morpholine N-oxide with dinitromethyl substances of different origin, the reaction of 2-(1,3-diphenyl-1H-1,2,4-triazol-5-yl)-2,2-dinitroaceto-nitrile 1 with morpholine N-oxide 2 was studied.

It was found that this interaction takes place in toluene medium with the addition of hydrogen peroxide and sodium tungstate to the reaction mixture, in quantities leading to the synthesis of 2-[(1,3-diphenyl-1H-1,2,4-triazol-5-yl)dinitromethyl]-5,6,8,8a-tetrahydro-[1,2,4]
oxadiazole[3,2-c][1,4]oxazine 3 with a yield of 42% (scheme).

Also, a resinous unidentified substance was additionally isolated from the reaction mixture, with a yield of 34%. It can be assumed that morpholine It is possible to assume that N-oxide of morpholine **A** is generated in the interaction of morpholine **2** with hydrogen peroxide in the presence of sodium tungstate in catalytic amounts [8, 9]. N-Oxide of morpholine A is stabilized into 2-[(1,3-diphenyl-1H-1,2,4-triazol-5-yl)dinitromethyl]-5,6,8,8a-tetra-hydro-[1,2,4]oxadiazole [3,2-c][1,4]oxazine **3** by the process of 1,3-dipolar cycloaddition to the dipolarophile molecule 1.



Ht=2-(1,3-diphenyl-1,2,4-triazol-5-yl)

Scheme. Synthesis of 2-[(1,3-diphenyl-1H-1,2,4-triazol-5-yl)dinitromethyl]-5,6,8,8a-tetrahydro-[1,2,4] oxadiazole[3,2-c][1,4]oxazine 3 and its alkaline cleavage reaction.

Elemental analysis showed the composition of compound 3 and the structure of this compound was studied using the sum of different methods - 1H NMR, 13C, mass spectrometry, IR spectroscopy and chemical transformations. IR spectroscopy for the original dinitroacetonitrile had characteristic absorption bands at 2255 cm^{-1} nitrile group, while cycloadduct **3** had none in its IR spectrum [10]. The 1H and 13C NMR spectra are proportional to those of a number of compounds of 1,2,4-oxadiazolines [11]. In the region of 3.12 ppmthe signal of the carbon atom C3 was fixed in the ^{1}H NMR spectrum of the oxadiazoline cycle, in the original compound there was no signal in the corresponding region. On the ^{13}C NMR spectrum at 87.4 ppma signal of the same carbon atom was detected. There was a low-power peak of the molecular ion and $[M-1]^+$ fragment in the mass-spectral analysis of the whole compound, and peaks characteristic of the "retro-1,3-dipolar cycloaddition" type interaction of the dissociative ionization process ions at the $O^{1'}$-$N^{2'}$ and
$C^{3'}$-$N^{4'}$oxadiazoline ring bonds were also recorded.

The interaction of compound 3 with an alcoholic solution of potassium hydroxide proceeds due to the electrophilicity of the compound. The process of salt formation of the potassium salt aci-5-dinitromethyl-1,3-diphenyl-1H-1,2,4-triazole **4** and 5,6,8,8a-tetrahydro-[1,2,4] oxadiazole[3,2-c][1,4]oxazine-2-ol **5** occurs under mild conditions with the cleavage of the 1,2,4-oxadiazoline cycle (scheme). The yield of compound 4 was 65%, and compound **5** was 14%. The mechanism of salt formation is based on the presence of two groups in compound **3**, strongly electron-acceptor and placed side by side, due to this, the bond strength decreases and the

C^5 atom is desecrated by the oxadiazoline heterocycle and the carbon of the dinitromethyl group.

2-[(1,3-Diphenyl-1H-1,2,4-triazol-5-yl)dinitromethyl]-5,6,8,8a-tetrahydro-[1,2,4]oxadiazole[3,2-c][1,4]oxazine 3. 5 mg of sodium tungstate was added to the mixture of substances (morpholine **2**, 50% solution of hydrogen peroxide, dried toluene) with vigorous stirring at 20°C. After intensive ten-minute mixing, 5 mmol of compound 1 was added to the mixture and the mixture was kept at 60° C for 24 hours. After evaporation of the solvent under vacuum conditions, the dry residue was chromatographed on a descending glass column (10 × 500 mm, grade Silicagel 100/400μ), with an increase in the eluting strength of the solvents of the Trappe series. Benzene was used as an eluent for compound 3. Pale yellow crystals were obtained, the yield was 0.9471 g (42%), melting point 132–135°C. ^{1}H NMR spectrum, δ, ppm: 3.12 t (1H, $C^{3'}$H), 2.95-3.78 m (6Hmorph., $3CH_2$), 7.48-7.72 m ($10H_{arom.}$, $2C_6H_5$). 13C NMR spectrum, δ, ppm: 54.2-65.4 ($C_{morph.}$), 142.1 (C^3), 129.2-136.8 ($C_{arom.}$), 87.4 ($C^{3'}$), 170.4 ($C^{5'}$), 122.4 (C^6), 155.7 (C^5). IR spectrum, ν, cm-1: 1580, 1300 (NO_2).

Mass spectrum, *m/z* (Irel., %): 451 (10) $[M]^+$, 450 (8) $[M-1]^+$, 46 (42), 30 (100). Found, %: C 53.04; H 3.58; N 21.52. $C_{20}H17N_7O_6$. Calculated, %: C 53.22; H 3.77; N 21.73.M 451.38.

Potassium salt of aci-5-dinitromethyl-1,3-diphenyl-1H-1,2,4-triazole 4 and 5,6,8,8a-tetrahydro-[1,2,4]oxadiazole[3,2-c] [1,4]oxazin-2-ol 5. To a mixture of compound 3 with ethanol, an excess of a saturated alcoholic solution of KOH is added dropwise to pH 9 at a temperature of 0ºC, then the reaction mixture is kept at a temperature of 20ºC for 1 h, as a result, compound 4 is obtained in the precipitate.

Salt 4 is filtered off and recrystallized from ethanol. The yield was 0.4709 g (65%), yellow crystals were obtained with a decomposition temperature of 285ºC [12]. After removal of the solvent, chromatography was carried out in the above manner, compound 5 was obtained, the eluent was diethyl ether. The product yield was 0.0402 g (14%), compound 5 is colorless crystals with a melting point of 162–165°C (ethanol). IR spectrum, ν, cm^{-1}: 3480 (OH). 1H NMR spectrum, δ, ppm: 6.72 br (1H, OH), 3.09 t (1H, C3H), 2.92–3.74 m ($6H_{morph}$, 3CH2). 13C NMR spectrum, δ, ppm: 168.8 (C^5), 86.2 (C^3), 54.2–65.4 ($C_{morph.}$). Found, %: C 41.52; H 5.39; N 19.28. $C_5H_8N_2O_3$. Calculated, %: C 41.67; H 5.56; N 19.44.

EXPERIMENTAL BIOLOGICAL PART

Materials and methods

The activity of the compounds was studied against strains of *St. aigeas* RP, *S. pneumoniae* (gram-positive bacteria), *E. coli O39, Ps. aeruginosa 143* (gram-negative bacteria).

Test cultures of fungi Microsporum canis 1/81 and Trichophyton rubrum 11/33, Candida albicans SS were used.

Microbiological studies were carried out using the method of direct diffusion into agar (ADM), this method was used to study the antimicrobial activity of various compounds in vitro. The essence of the method is as follows: on Petri dishes with meat-peptone agar, half an hour before the introduction of the studied compounds, a test culture was sown (in 1 ml of isotonic solution - 105 microbial bodies). The studied substances, previously diluted in dimethyl sulfoxide (DMSO), were introduced into wells on a nutrient medium at concentrations of 1 mg/ml of 25 μl. The cups were left for incubation for a day at a temperature of 37±1°C in a thermostat. The next day, the diameter of the growth retardation zones (DZR) of test strains around the well was measured (with an accuracy of ±1 mm). Gentamicin, as a broad-spectrum antibiotic and a drug to which our test strains of microorganisms showed increased sensitivity, was used as a comparison drug, it was also pre-mixed with DMSO at a dose of 40 mg/ml.

After studying the activity by diffusion into agar, we determined the minimum suppressive concentration (ADM) of our compounds by double serial dilutions. In this technique, a dilution technique is used, in which the dose of the active substance was halved in each new dilution (in each subsequent test tube). Tables 1 and 2 show the results of the antibacterial activity of the studied compounds, which were subjected to statistical processing.

The study of antifungal activity under *in vitro* conditions was carried out using the method of serial dilutions in a liquid and dense Saburo medium. Test cultures of Microsporumcanis 1/81 and *Trichophyton rubrum 11/33 fungi, Candida albicans* SS were used in the work. The method used the same dilution technique to obtain the specified dosages of active substances in each subsequent test tube. The minimum fungistatic concentration was taken to be one at which a 50% decrease in growth was observed. Flucanazole, as a first-line drug in the treatment of fungal infections, was chosen as a comparison drug.

Table 1. Results of a comparative study of antibacterial activity synthesized compounds and gentamicin by disk-diffusion method

Compound	Diameter of growth retardation zones, mm			
	Staphylococcus aureus RP	*Streptococcus pneumoniae*	*Escherichia coli O39*	*Pseudomonas aerugi nosa 143*
3	46±3,2	40±3,1	$35±1,8^{\Delta\Delta\Delta}$	$40±1,4^{***}$
4	46±0,8	40±3,2	$34±1,6^{\Delta\Delta\Delta}$	$39±2,8^{***}$
5	$10±0,1^{***}$	0^{****}	$8±2,5^{***}$	$11±1,2^{***}$

Gentamicin	42±1,5	40±0,7	22±0,4	34±0,5

$^{\Delta\Delta\Delta}p \leq 0,001$ compared to gentamicin (activity higher), $^{***}p \leq 0,001$ compared to gentamicin (activity lower).

Table 2. Concentration ranges of synthesized growth-inhibiting compounds pathogen by 50% compared to gentamicin (serial dilution method)

Compound	Minimal inhibitory concentration (MIC_{50}), mcg/ml			
	Staphylococcus aureus RP	*Streptococcus pneumoniae*	*Escherichia coli O39*	*Pseudomonas aeruginosa 143*
3	3,9±0,5	3,9±0,5	7,8±0,2**	7,8±0,2**
4	7,8±0,09**	7,8±0,2**	3,9±0,1	7,8±0,2**
5	31±0,8***	62,5±0,2***	31±0,7***	31,2±0,9**
Gentamicin	4,3±0,16	4,2±0,12	4,0±0,11	4,3±0,3

$^{**}p \leq 0,01$ compared to gentamicin, $^{***}p \leq 0,001$ compared to gentamicin.

Five series of experiments were conducted. A fungal suspension was added to the samples with dilutions of drugs mixed with DMSO, then the tubes were placed in a thermostat for 7 days at a temperature of 24±3°C (Candida albicans) and for 30 days at a temperature of 24±3°C (*Trichophyton rubrum* and *Microsporum canis*). After incubation, the minimum fungistatic concentration (MFC) of the studied substances was visually determined by the degree of inhibition of the growth of test cultures of fungi. For a more accurate determination of the IFC, the material was seeded from each tube onto agar wort in Petri dishes. The cups were placed in a ter-mostate at 24±3 ° C for 30 days (*Microsporum canis* and *Trichophyton rubrum*) and 7 days (*Candida albicans*) [14].

Results and discussion

The compounds 3-5 we obtained were examined for antimicrobial activity against a number of museum cultures such as *Staphylococcus aureus RP, Escherichia coli O39, Pseudomonas aeruginosa 143* and a clinical strain of *Streptococcus pneumonia*.

The data presented in Table 1 indicate that compounds 3 and 4 in relation to the *Staphylococcus aureus RP* strain have a pronounced activity. The growth retardation zone (GDZ) of this microorganism was comparable in magnitude to the GDZ with gentamicin, which was the reference drug. Compound 5 showed no activity (Table 1).

Identical action of the compounds was revealed with respect to the strain *Streptococcus pneumoniae* used: the growth retardation zones under the action of compounds 3 and 4 did not differ in magnitude from the control ones. Compound 5 was absolutely indifferent (Table 1).

In relation to the museum strain Escherichia coli O_{39}, substances 3 and 4 showed pronounced activity: the zones of growth inhibition when these compounds were introduced into nutrient media were significantly wider than when using the reference drug (by 59% and 54%, respectively). The effect of compound 5 was weak (Table 1).

The ability of compounds 3 and 4 to inhibit the growth of the Pseudomonas aeruginosa 143 strain was also pronounced and exceeded the effect of gentamicin: the growth inhibition zones of this bacterium were statistically significantly wider compared to the reference drug. Compound 5 was significantly inferior in action to the control (Table 1).

The activity of the studied compounds against strains was confirmed in the second series of experiments (in series of serial dilutions) when studying their minimum inhibitory concentration (Table 2).

As shown in Table 2, with respect to Gram-positive microorganisms, the minimum suppressive concentration of compound 3 was comparable with that of gentamicin. Compound 4 was superior to it in its ability to suppress the growth of these test strains and exceeded the MSC of gentamicin twofold, but remained steadily low, which corresponded to the requirements for antibiotics.

Compound 4 turned out to be the most active against Gram-negative strain E.coli O39 with MSC=3.9 μg/ml. In compound 3 this index was twice as high, but remained steadily low, as in the previous case.

The ability of compounds 3 and 4 to inhibit the growth of Gram-negative strain *Pseudomonas aeruginosa 143* was identical and, despite their MIC being higher than that of the reference preparation, was within the range of effective concentrations.

The MSC of compound 5 was significantly, statistically significantly higher than both the minimum suppressive concentration of gentamicin and compounds 3 and 4 against all test strains and, accordingly, was less effective (Table 2).

Continuing our search for substances with antifungal activity, we studied the activity of synthesized compounds 3-5 against a number of pathogenic fungi.

The results are presented in Table 3.

Table 3. Results of a comparative study of antifungal activity synthesized gentamicin compounds (serial dilution method)

Compound	Minimum fungistatic concentration (MFK_{50}), mcg/ml

	Candida albicans SS	*Microsporum canis1/81*	*Trichophyton rubrum 11/33*
3	80±9,4*	80±9,5*	80±9,5*
4	160±18,9**	160±18,9**	160±19,0**
5	320±37,9**	320±38,1**	320±38,5**

*p≤0,05 regarding fluconazole, **p≤0,01 regarding fluconazole.

Compounds **3**-**5** showed different fungistatic activity against test strains of fungi with a wide range of concentrations: from 40 to 320 μg/ml. The leader is compound **3**, which has a minimum inhibitory concentration, which is significantly lower in comparison with compounds **4** and **5** in relation to all studied fungal species. In all cases, the minimum fungistatic concentration of the compounds was statistically significantly higher than that of the reference drug fluconazole.

Thus, as a result of the "*one pot*" process (the reaction of 2-(1,3-diphenyl-1H-1,2,4-triazol-5-yl)-2,2-dinitroacetonitrile 1 with morpholine 2 in the presence of sodium tungstate and hydrogen peroxide) it is possible to insert oxadiazoline and oxazine rings into the main (central) part of the molecule of compound **1**, which leads to the synthesis of new agents with antimicrobial and antifungal activity.

AUTHOR'S CONTRIBUTION

Each author participated in the implementation of experimental work, interpretation of analytical data, discussion and generalization of the results. The share of each author's contribution to the article is 0.25.

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