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Synthesis and Antimicrobial and Toxic Properties of Novel 1,3-Bis(alkyl)-6-Methyluracil Derivatives Containing 1,2,3- and 1,2,4-Triazolium Fragments[1]

A. D. Voloshina[2], V. E. Semenov, A. S. Strobykina, N. V. Kulik, E. S. Krylova, V. V. Zobov, and V. S. Reznik

Arbuzov Institute of Organic and Physical Chemistry, Kazan Research Center, Russian Academy of Sciences, Kazan, 420088 Russia

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Abstract—The antimicrobial activity and cytotoxicity of novel 1,3-bis(alkyl)-6-methyluracil derivatives containing 1,2,3- and 1,2,4-triazolium fragments in alkyl chains have been studied. The compounds have been tested for the antimicrobial activity toward some gram-positive and gram-negative bacteria and fungal cultures. The cytotoxic action has been estimated toward mammalian cells. It has been found that the basic structural factor that affects the antimicrobial activity is the nature of alkyl radicals at triazole fragments.

Keywords: triazoles, uracils, antimicrobial activity, cytotoxicity, hemolytic activity

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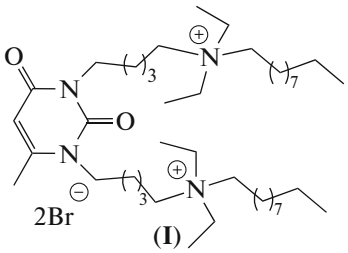
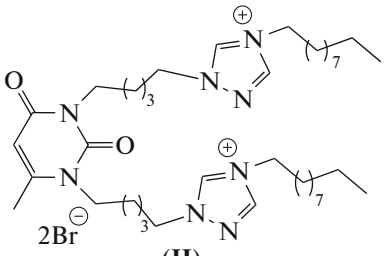
INTRODUCTION

In view of the rapid acquisition of drug resistance by pathogenic microorganisms, a search for antimicrobial nontoxic medicinal agents having radically new mechanisms of action is of prime importance. It has been shown earlier that 1,3-bis(alkyl)-6(5)-substituted uracil derivatives containing onium groups in polymethylene chains, in particular, compound (**I**), exhibit a wide spectrum of antimicrobial activity, low hemolytic activity, and moderate toxicity in mammals [1–5].

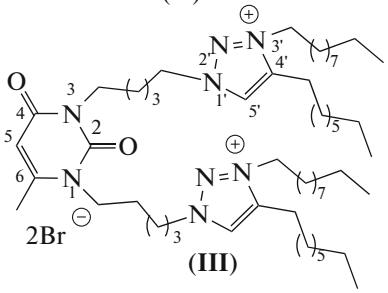
The derivatives of 1,2,3- and 1,2,4-triazoles are used as biologically active substances of different action. They possess antibacterial, antifungal, antitumor, and anti-inflammatory properties [6–8]. In the present work, 1,3-bis(alkyl)-6-methyluracil derivatives containing 1,2,4-triazolium (**II**) and 1,2,3-triazolium fragments (**III**) in alkyl chains were tested for the antimicrobial activity and cytotoxicity for the first time. Compound (**II**) has been synthesized earlier [9], and compound (**III**) was synthesized for the first time.



(**I**)



(**II**)



(**III**)

RESULTS AND DISCUSSION

Synthesis of 1,3-bis(alkyl)-6-methyluracil derivatives. Compound (**I**) was synthesized by the quater-

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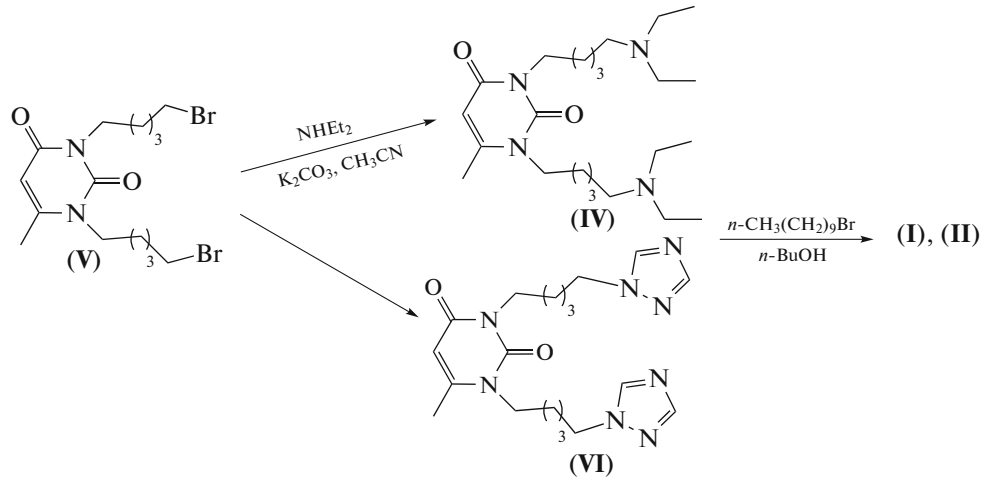
[2] Corresponding author: phone: +7 (917) 262-71-90; e-mail: microbi@iopc.ru.

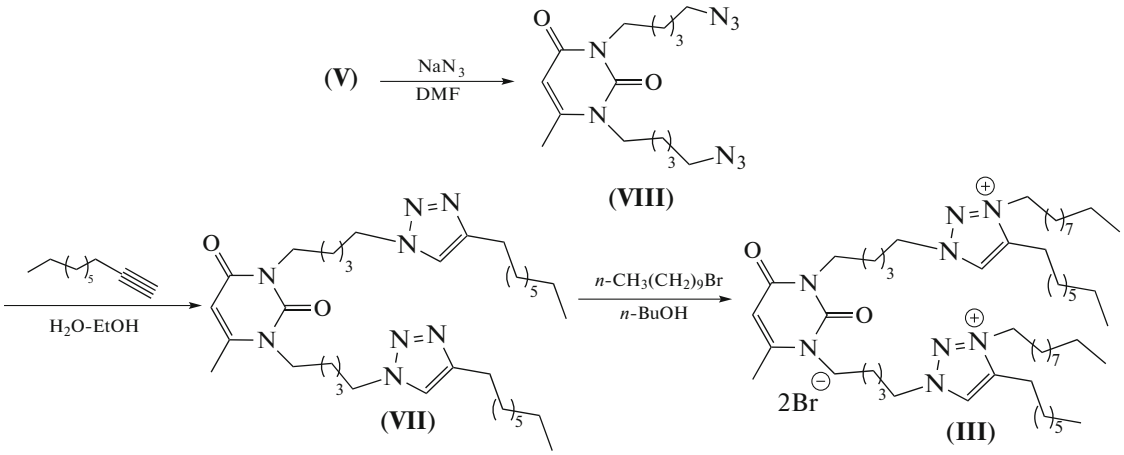
Abbreviations: MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; MFC, minimal fungicidal concentration.

nization of N atoms in alkyl chains of 1,3-bis(5-aminoethylpentyl)-6-methyluracil (**IV**) by *n*-decyl bromide as described in [1, 2] (Scheme 1). Compound (**II**) was obtained by the conventional method by the reaction of 1,3-bis(5-bromopentyl)-6-methyluracil (**V**) with 1,2,4-triazole followed by the alkylation of the N atom of the 1,2,4-triazole ring of the resulting 1,3-bis[5-(1,2,4-triazole-1-yl)pentyl]-6-methyluracil (**VI**) with *n*-decyl bromide [9] (Scheme 1).



Scheme 1. Synthesis of compounds (**IV**) and (**I**) [1, 2] and of compounds (**VI**) and (**II**) [10].

Compound (**III**) was synthesized by alkylating 1,3-bis[5-(4-octyl-1,2,3-triazol-1-yl)pentyl]-6-methyluracil (**VII**) with *n*-decyl bromide. The sites of alkylation of 1,2,3-triazole rings in compound (**VII**), namely, N3' atoms, were identified using 1D and 2D NMR experiments (^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C/^{15}N HSQC, HMBC, ROESY, and NOESY). Compound (**VII**) in turn was synthesized as described in [10] by substituting the azide groups for bromine atoms in dibromide (**V**) and introducing the resulting compound 1,3-bis(5-azidopentyl)-6-methyluracil (**VIII**) into the 1,3-cycloaddition reaction with 1-decine [10] (Scheme 2).

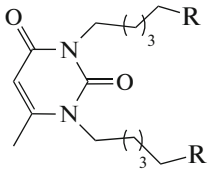


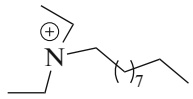
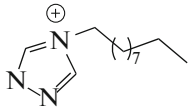
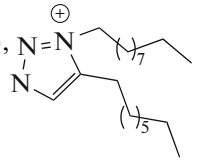
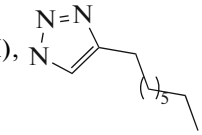
Scheme 2. Synthesis of compounds (**VIII**) and (**VII**) [10] and of compound (**III**).

Antimicrobial activity of 1,3-bis(alkyl)-6-methyluracil derivatives. The activity of the compounds synthesized was tested toward some Gram-positive (*Staphylococcus aureus* 209-P, *Bacillus cereus* 8035) and Gram-negative bacteria (*Pseudomonas aeruginosa* 9027, *Escherichia coli* F-50) and fungi (*Trichophyton mentagrophytes* var. *gypseum* 1773, *Aspergillus niger* BKMF-1119, *Candida albicans* 885-653). The results are given in Tables 1 and 2 in terms of MICs (concentrations that stop bacterial and fungal growth) and MBCs and MFCs (minimal concentrations inducing the cell death).

Compounds (**I**)–(**III**) have some common structural features, namely, five methylene groups in bridging chains and an *n*-decyl radical in the composition of the onium group [compound (**I**)] and triazole rings

Table 1. Bacteriostatic and fungistatic activities of 1,3-bis(alkyl)-6-methyluracil derivatives containing 1,2,3- and 1,2,4-triazole and -triazolium fragments in alkyl chains, expressed in terms of MIC



Compound, R	MIC, μg mL^{-1} (μM)						
	Sa	*Bc*	*Ec*	*Pa*	*An*	*Tm*	*Ca*
(I), 	1.6 ± 0.1 (1.9 ± 0.1)	2.5 ± 0.2 (2.9 ± 0.2)	12.5 ± 1.2 (14.7 ± 1.4)	–	62.5 ± 5.3 (73.7 ± 6.2)	31.3 ± 2.9 (36.9 ± 3.4)	31.3 ± 2.7 (36.3 ± 3.1)
(II), 	2.0 ± 0.2 (2.4 ± 0.2)	4.0 ± 0.4 (4.8 ± 0.7)	8.0 ± 0.7 (9.5 ± 0.8)	125 ± 11 (148 ± 13)	125 ± 12 (148 ± 14)	3.1 ± 0.3 (3.6 ± 0.3)	0.80 ± 0.07 (0.90 ± 0.08)
(III), 	15.6 ± 1.5 (14.7 ± 1.4)	62.5 ± 5.5 (58.7 ± 5.2)	62.5 ± 5.8 (58.7 ± 5.4)	500 ± 45 (470 ± 42)	250 ± 22 (235 ± 21)	6.3 ± 0.6 (5.9 ± 0.5)	6.3 ± 0.5 (5.9 ± 0.5)
(IV), NEt_2	–	–	–	–	–	–	–
(VI), 	–	–	–	–	–	–	–
(VII), 	31.3 ± 2.8 (50.1 ± 4.5)	62.5 ± 5.9 (100 ± 9.5)	–	–	–	–	–
Norfloxacin	2.4 ± 0.2 (7.5 ± 0.6)	8.0 ± 0.7 (25.0 ± 2.2)	1.5 ± 0.1 (4.7 ± 0.3)	3.0 ± 0.3 (9.4 ± 0.9)	–	–	–
Chloramphenicol	(62.5 ± 5.8) (193 ± 18)	62.5 ± 5.6 (193 ± 18)	125 ± 12 (386 ± 37)	–	–	–	–
Ketoconazole	–	–	–	–	4.0 ± 0.4 (7.6 ± 0.7)	4.0 ± 0.3 (7.6 ± 0.5)	4.0 ± 0.3 (7.6 ± 0.5)

Sa, *Staphylococcus aureus*; *Bc, Bacillus cereus*; *Ec, Escherichia coli*; *Pa*, *Pseudomonas aeruginosa*; *An*, *Aspergillus niger*; *Tm, Trichophyton mentagrophytes; Ca, Candida albicans*; MIC > 500 μg mL^{-1}.

Table 2. Bactericidal and fungicidal activities of derivatives (**I**–**III**), expressed in terms of MBC and MFC

Compound	MBC (MFC), μg mL^{-1} (μM)*						
	Sa	*Bc*	*Ec*	*Pa*	*An*	*Tm*	*Ca*
(I)	500 ± 44 (590 ± 52)	–	–	–	–	–	–
(II)	50.0 ± 4.4 (59.0 ± 5.2)	–	–	–	–	31.3 ± 2.8 (37.2 ± 3.3)	50.0 ± 4.5 (59.5 ± 5.4)
(III)	50.0 ± 4.8 (47.0 ± 4.5)	–	–	–	–	125 ± 11 (117 ± 10)	50.0 ± 4.3 (47.0 ± 4.0)

* For *Bc*, *Ec*, *Pa*, and *An*, MBC (MFC) > 500 μg mL^{-1} (see Table 1).

[compounds (**II**) and (**III**)]. By introducing the triazolium fragments containing an *n*-decyl radical into the molecule of a 1,3-bis(alkyl)-6-methyluracil derivative and based on the literature data and the results of our investigations [1–7], we hoped to obtain compounds with high antifungal and antibacterial activities.

The data in Table 1 show that, similar to the earlier described 6-methyluracil onium derivative (**I**), 1,3-bis(alkyl)-6-methyluracil derivative (**II**), which contains 1,2,4-triazolium fragments in alkyl chains, exhibits bacteriostatic activity toward Gram-positive bacteria (*S. aureus* 209-P and *B. cereus* 8035) similar to the activity of the antibiotic Norfloxacin from the series of fluoroquinolones and a higher activity, as compared to compound (**I**), toward Gram-negative bacteria (*P. aeruginosa* 9027 and *E. coli* F-50).

The fungistatic activity of compound (**II**) toward *T. mentagrophytes* var. *gypseum* 1773 and *C. albicans* 885-653 is substantially higher than that of both compound (**I**) and the reference preparation Ketoconazole. 1,3-Bis(alkyl)-6-methyluracil derivative (**III**), which contains 1,2,3-triazolium fragments, showed a lower activity towards bacteria compared to compounds (**I**) and (**II**), which is nevertheless comparable with the MIC of the reference preparation chloramphenicol. However, the MIC of compound (**III**) toward *T. mentagrophytes* var. *gypseum* 1773 and *C. albicans* 885-653 is fivefold lower than the MIC of the 6-methyluracil onium derivative (**I**) but is higher than that of compound (**II**) and the reference preparation Ketoconazole toward these fungi. The high fungistatic activity of compounds (**II**) and (**III**) is probably related to the specific influence of triazolium fragments introduced into pentamethylene chains.

Thus, the quaternization of aliphatic nitrogen atoms and nitrogen atoms of triazole rings in alkyl chains at the 6-methyluracil fragment is of critical importance for the manifestation of antimicrobial properties. For instance, compounds (**IV**), (**VI**), and (**VII**) whose alkylation with *n*-decyl bromide led to compounds (**I**)–(**III**) are almost inactive, and only compound (**VII**) exhibits some activity toward Gram-positive bacteria (Table 1). The introduction of the *n*-decyl radical sharply increases their antimicrobial activity. In addition, the introduction of 1,2,4-triazolium fragments carrying the *n*-decyl radical [compound (**II**)] at N atoms of the 6-methyluracil cycle substantially enhances the antifungal activity compared with the activity of 6-methyluracil onium derivative (**I**), whereas the introduction into the alkyl chains of 1,2,3-triazolium fragments carrying, along with the *n*-decyl radical, an octyl substituent [compound (**III**)] significantly decreases antibacterial properties.

Table 2 gives the values of MBC and MFC of the compounds under study. It is seen that, as opposed to compound (**I**), compounds (**II**) and (**III**) exhibit bactericidal activity toward *S. aureus* 209-P and fungicidal activity toward *T. mentagrophytes* var. *gypseum* 1773 and *C. albicans* 885-653.

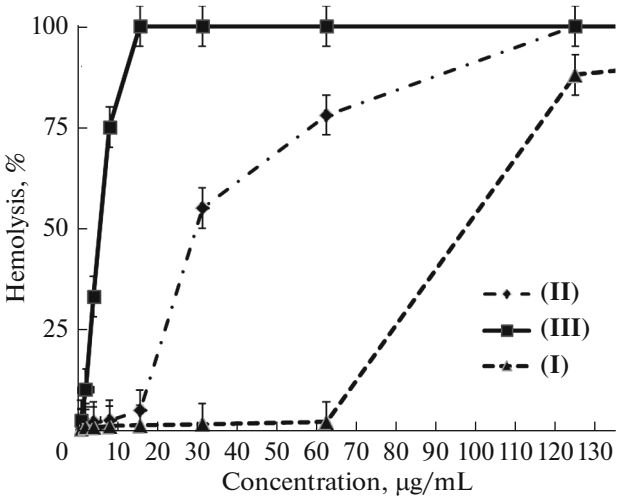


Fig. 1. Hemolytic activity of derivatives (**I**)–(**III**).

An important characteristic in the development of novel drugs is their cytotoxic action on mammalian cells. 1,3-Bis(alkyl)-6-methyluracil derivatives containing 1,2,3- and 1,2,4-triazolium fragments were tested for cytotoxicity on human erythrocytes (hemolytic activity) [1] and WI-38 cell culture (human embryonic lung).

In Fig. 1 the data are shown on the hemolysis of human erythrocytes, induced by the compounds being examined and by the earlier-described onium derivative of 6-methyluracil (**I**). The hemolysis of erythrocytes in the presence of compound (**II**) in the range of MICs (0.8—8.0 μg mL^{-1}) does not exceed 2%, whereas compound (**III**) has a rather high hemolytic activity. The degree of hemolysis at the lowest concentration (6.3 μg mL^{-1}), which inhibits the growth of test fungal strains, was 56%. Compounds (**II**) and (**III**) were found to be more toxic toward human erythrocytes compared with (**I**).

Figure 2 shows the data on the cytotoxic action of 1,3-bis(alkyl)-6-methyluracil derivatives (**II**) and (**III**) on the WI-38 cell culture. The cytotoxicity was estimated by counting viable cells during cultivation with the compounds at concentrations corresponding to the MICs determined for the bacterial and fungal test strains (Table 1). These concentrations were 0.8, 2, 4, and 8 μg mL^{-1} for compound (**II**) and 6.3 and 15.6 μg mL^{-1} for (**III**).

An analysis of the results showed that compound (**II**) was least toxic toward human embryonic lung cells. The cell viability calculated in percent of the control was 77% at a MIC of 0.8 μg mL^{-1} for the fungus *C. albicans* and 46% at a MIC of 2.0 μg mL^{-1} for the bacteria *S. aureus*. Compound (**III**) was found to be more toxic toward WI-38 cells. The survival of cells at

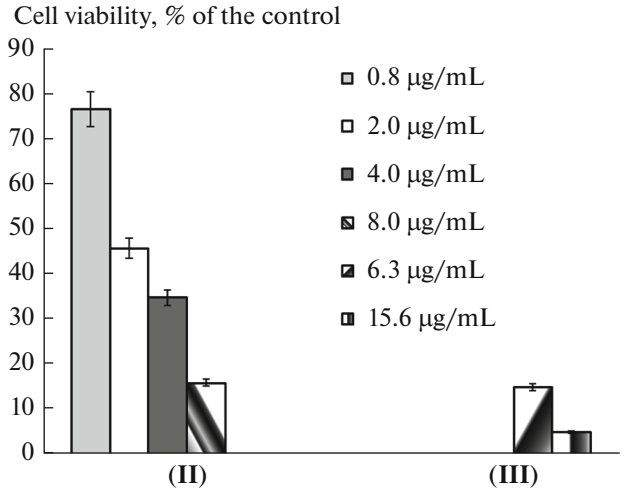


Fig. 2. Cytotoxic activity of compounds (**II**) and (**III**) toward the WI-38 cell culture.

the operation concentrations of 15.6 and 6.3 μg mL^{-1} was 5 and 15%, respectively.

We have shown earlier that the onium derivatives of 1,3-bis(alkyl)-6-methyluracil inhibit the activity of glucose dehydrogenases in *S. aureus* 209-P and *C. albicans* [1, 4]. We estimated the effect of 1,3-bis(alkyl)-6-methyluracil derivatives containing 1,2,4- and 1,2,3-triazolium fragments in alkyl chains on the activity of these enzymes. The results (Table 3) showed that these compounds, similar to (**I**), have a pronounced effect on the activity of dehydrogenases in *S. aureus* 209-P and *C. albicans*. Compound (**II**) inhibits the activity of this enzyme to a greater extent than (**I**) and (**III**). Thus, at a concentration of 5 μg mL^{-1}, compound (**II**) inhibits the dehydrogenase activity in *S. aureus* by 58% and in *C. albicans* by 65%, whereas compound (**I**) inhibits the activity of these enzymes by 61 and 48%, respectively, and compound (**III**), by 55 and 38%, respectively.

Based on these results, it can be assumed that the mechanism of action of the compounds tested is associated with the inhibition of the enzyme systems of the respiratory chain of microorganisms at early stages of interaction with cell targets, which disturbs the normal course of the synthesis of vital compounds in the cell of a microorganism.

Thus, the 1,3-bis(alkyl)-6-methyluracil derivatives containing 1,2,3- and 1,2,4-triazolium fragments have a marked bacteriostatic activity. In particular, the minimal bacteriostatic concentration of compound (**II**) for *S. aureus* bacteria is 2.0 μg mL^{-1}, and the minimal fungistatic concentration for *C. albicans* is 0.8 μg mL^{-1}. We determined a specific contribution of 1,2,3- and 1,2,4-triazolium fragments to the antimicrobial effect of the compounds: the introduction of 1,2,3- and 1,2,4-triazolium fragments carrying the *n*-decyl radical into the pentametylene chains at N atoms of the 6-methyluracil cycle [compound (**II**)] significantly enhances the antifungal activity as compared with the onium derivative of 6-methyluracil (**I**), whereas the introduction of 1,2,3-triazolium fragments carrying, along with the *n*-decyl radical, an octyl substituent into alkyl chains [compound (**III**)] considerably diminishes the antibacterial properties and increases the cytotoxicity. In the range of concentrations that stop the growth of bacteria and fungi, compound (**II**) does not exhibit high cytotoxicity toward human erythrocytes and WI-38 culture cells.

The mechanism of the antimicrobial action of 1,3-bis(alkyl)-6-methyluracil derivatives containing 1,2,3- and 1,2,4-triazolium fragments is probably related to the inhibition of the enzyme systems of the respiratory chain and energy exchange in microorganisms.

EXPERIMENTAL

1D- and 2D NMR experiments (^1H, ^{13}C) with compound (**III**) were performed on an AVANCE-500 Fourier spectrometer (Bruker) at a working frequency of 500.13 MHz (^1H) and 125.77 MHz (^{13}C) in $CDCl_3$ at 30°C with tetramethylsilane as an internal standard. IR spectra of compound (**III**) were recorded in a thin layer on a Vector 22 Fourier spectrometer (Bruker) under standard conditions in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1}. MALDI-TOF mass spectra were recorded on an UltraFlex III mass spectrometer (Bruker). The elemental analysis was carried out on a EuroVector CHN analyzer.

1,3-Bis[5-(3-*n*-decyl-4-octyl-1,2,3-triazolium-1-yl)pentyl]-6-methyluracil dibromide (III). *n*-Decyl bromide (0.8 g, 4 mmol) was added to a suspension (0.50 g, 0.8 mmol) of 1,3-bis[5-(4-octyl-1,2,3-triazol-1-yl)pentyl]-6-methyluracil (**VII**), obtained by the method described in [10], in *n*-BuOH. The reaction mass was stirred at 100—110°C for 20 h. The course of the reaction was monitored by TLC. After the cooling of the reaction mixture, the solvent was evaporated in vacuo, and diethyl ether was added to the residue and decanted. The procedure was repeated three times. The residue was dried in a vacuum of a water-jet pump. The yield of compound (**III**) as an oil: 0.73 g (86%). IR, ν_{max}, cm^{-1}: 2926, 2856, 1698, 1658, 1620, 1577, 1465, 1432, 1403, 1362, 1209, 1118, 1058, 815, 770, 724, 627, 547; ^1H NMR: 9.77 (br s, 2H, 2H5'), 5.53 (s, 1H, H5), 4.91–4.84 (m, 4H, 2 N3'CH_2), 4.51–4.43 (m, 4H, 2N1'CH_2), 3.98–3.87 (m, 4H, N1CH_2, N3CH_2), 2.89–2.80 (m, 4H, 2C4'CH_2), 2.30 (s, 3H, C6CH_3), 2.04–1.95 (m, 8H, 4CH_2), 1.83–1.72 (m, 8H, 4CH_2), 1.46–1.28 (m, 52H, 26CH_2), 0.94–0.85 (m, 12H, 4CH_3); ^{13}C NMR, {^1H}, δ, ppm: 162.0, 152.5, 143.8, 130.0, 129.8, 101.7, 52.1, 51.7, 42.2, 37.5, 31.5, 29.0, 28.8, 27.5, 26.4, 25.9, 23.7, 22.3, 21.8, 20.6, 13.8, 13.5; MALDI-TOF mass spectrum, *m/z*: 907.0 [*M*-2Br]$^+$, 765.4 [M-2Br-$C_{10}H_{21}$]$^+$; calculated for

Table 3. Inhibition of the activity of glucose dehydrogenase (DH) of *S. aureus* 209-P and *C. albicans* 885-653 (**I**–**III**)

Compound	Concentration μg mL^{-1}	Inhibition of DH activity, %	
		S. aureus	*C. albicans*
(I)	500 ± 46	100 ± 9	100 ± 8
	50.0 ± 4.4	68 ± 6	100 ± 9
	5.0 ± 0.5	61 ± 5	48 ± 4
	0.50 ± 0.04	56 ± 5	0
(II)	500 ± 45	100 ± 9	100 ± 9
	50 ± 4.5	72 ± 6	78 ± 7
	5.0 ± 0.4	58 ± 6	65 ± 6
	0.50 ± 0.05	0	0
(III)	500 ± 49	100 ± 8	77 ± 7
	50.0 ± 4.7	68 ± 6	45 ± 4
	5.0 ± 0.4	55 ± 5	38 ± 3
	0.50 ± 0.05	0	0

$C_{55}H_{102}N_8O_2Br_2$ 906.8, 765.6. Found, %: C, 61.82; H, 9.59; N, 10.63; Br, 15.12. $C_{55}H_{102}N_8O_2Br_2$. Calculated, %: C, 61.90; H, 9.63; N, 10.50; Br, 14.97.

The bacteriostatic activity of the compounds toward *P. aeruginosa* 9027, *E. coli* F-50, *S. aureus* 209-P, and *B. cereus* 8035 was determined by the method of two-fold serial dilutions [12] in a liquid nutrient medium. The bacterial load was 3.0×10^5 microbial bodies mL^{-1}. The results were recorded every 24 h for 5 days. Cultures were incubated at 37°C. The experiment was repeated twice. The fungistatic activity of aqueous solutions of the compounds toward the fungi *T. mentagrophytes* var. *gypseum* 1773, *A. niger* BKMF-1119, and *C. albicans* 885-653 was determined by the method of serial dilutions [13] on Sabouraud liquid medium. The time of the exposure in a thermostat at 25°C with the corresponding compound was 14 days. The dilutions of the compounds were prepared immediately in nutrient media; for better solubility, 5% DMSO was added, which does not induce the inhibition of test strains at this concentration. The MIC was defined as the minimal concentration of a compound that inhibits the growth of the corresponding test microorganism. The growth of bacteria and fungi as well as the absence of the growth owing to the bacteriostatic and fungistatic action of a compound were recorded. The bactericidal and fungicidal activities were determined as described earlier [5].

The hemolytic action of the compounds was estimated by comparing the optical density of a solution containing a compound being tested and the blood with the optical density of the blood upon 100% hemolysis. A 10% suspension of human erythrocytes was used as an object of investigation; an erythrocytic mass with heparin was washed three times with a physiological solution (0.9% NaCl), centrifuged for 10 min at 800 g, and resuspended in the physiological solution to a concentration of 10%. The concentrations of the compounds that corresponded to the MIC for the bacterial and fungal test strains were prepared in a physiological solution (supplemented with 5% DMSO), and 4.5 mL of a compound at the corresponding dilution was added to 0.5 mL of a 10% suspension of erythrocytes. Samples were incubated for 1 h at 37°C and centrifuged for 10 min at 2000 g. The release of hemoglobin was controlled by measuring the optical density of the supernatant on a digital photoelectrocolorimeter AP-101 (Apel, Japan) at λ 540 nm. Simultaneously, control samples were prepared: controls for zero hemolysis (blank): 0.5 mL of a 10% suspension of erythrocytes was added to a physiological solution; 100% hemolysis: 0.5 mL of a 10% suspension of erythrocytes was added to 4.5 mL of distilled water.

The cytotoxicity of the compounds at the MIC, which inhibits the growth of bacterial and fungal test strains, was estimated by counting the viable WI-38 cells, as compared with the control, by the Cytell Cell Imaging multifunctional system (GE Health Care Life Science, Sweden) using the Quick Count BioApp application, which makes it possible to precisely count the number of cells and estimate their viability from the fluorescence intensity with the use of disposable hemocytometer. The WI-38 VA 13 cell culture, subline 2RA (human embryonic lung), from the Collection of the Institute of Cytology (Russian Academy of Sciences) was used for experiments. Cells were cultured in a standard Eagle's nutrient medium manufactured at the Chumakov Institute of Poliomyelitis and Virus Encephalitis (PanEco company) and supplemented with 10% fetal calf serum and 1% nonessential amino

acids. WI-38 cells were plated into a 24-well plate (Eppendorf) at a concentration of 200000 cells/mL, 500 μL of medium per well, and cultured in a CO_2 incubator at 37°C. Twenty four hours after seeding the cells into wells, a compound examined was added at a preset dilution, 500 μL to each well. The dilutions of the compounds were prepared immediately in nutrient media; for better solubility, 5% DMSO was added, which does not induce the inhibition of WI-38 cells at this concentration. The experiments were performed in triplicates. Intact cells cultured in parallel with experimental cells served as a control.

The dehydrogenase activity in *S. aureus* 209-P and *C. albicans* 885-653 was determined under anaerobic conditions from the time of bleaching of methylene blue by the method of Thunberg [14, 15]. The cytometric results were analyzed by the Cytell Cell Imaging multifunctional system using the Quick Count BioApp application. Graphs were plotted using the program SigmaPlot, MS Excel. The data in the tables and graphs are the means plus standard errors of the mean.

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