4-Hydroxy-3-nitro-1,4-dihydrotriazolo[5,1-*c*][1,2,4]triazines: synthesis, antiviral activity, and electrochemical characteristics*

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A new method for preparation of 4-hydroxy-3-nitro-1,4-dihydrotriazolo[5,1-*c*][1,2,4]-triazines using 1-nitro-2-morpholinoethylene and 3-diazo-1,2,4-triazoles is proposed. Antiviral activity against the Coxsackie B3 virus and electrochemical transformations of the prepared compounds are studied.

Key words: 3-nitroazolo[5,1-*c*][1,2,4]triazines, azolotriazines, triazines, antiviral activity, Coxsackie B3 virus, voltammetry, electrochemical properties.

The urgency of the problem of creating drugs for antiviral therapy, especially, under conditions of rapid mutation of viruses and the identification of new pathogens of dangerous viral infections, indicates a constant need for new drugs with high activity, long-term action, and low toxicity. It should be noted that at present, despite a significant number of promising developments of direct-acting antiviral drugs, there are no unambiguously recognized and universally certified antivirals for the treatment of infections caused by enteroviruses.[1]

Group B coxsackieviruses belong to the *Enteroviridae* family of non-enveloped viruses with (+)-RNA genome and cause a wide range of pathologies, including peri- and myocarditis, poliomyelitis, nonspecific febrile fever, serous meningitis and meningoencephalitis, *etc*. In particular, the Coxsackie B3 virus is one of the main infectious agents that cause the development of viral myocarditis, the process that leads to inflammatory reactions in myocardial tissue and cardiomyocytic degeneration, causing heart failure. The coxsackievirus myocarditis presents exceptional difficulties for diagnosis and treatment, which is due to both clinical features (different degrees of severity of organic and functional damage to the myocardium, difficulties in obtaining material for biopsy, lack of clear markers of the disease), and the lack of effective etiotropic drugs.[2] Despite advances in the development of pharmacological drugs with antiviral activity, there are no drug that can effectively be used in etiotropic therapy against coxsackieviruses.

The main components of the drug therapy are symptomatic and pathogenetic drugs.

Given all of the above, the search for new compounds, as well as the study of the effectiveness of known antiviral drugs against the Coxsackie B3 virus, is one of the primary tasks of medicinal chemistry and virology.

Among the promising compounds possessing antiviral activity, 3-nitro-4-oxo-[1,2,4]triazolo[5,1-*c*]-[1,2,4]triazines are of particular interest.[3] It has been established that triazolo[5,1-*c*][1,2,4]triazines with an alkyl sulfanyl group at position 7 are most interesting.[4—7] In particular, dihydrate of sodium salt of 6-methylthio-3-nitro-1,2,4-triazolo[5,1-*c*][1,2,4]triazin-4-one (triazavirin, also known by the international non-proprietary name Riamilovir) has an antiviral effect against a number of RNA viruses and is approved for

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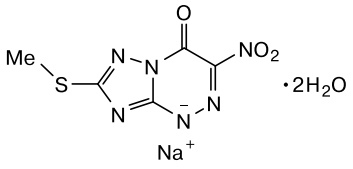
use in the treatment and prevention of influenza and SARS and also recommended for the treatment and prevention of COVID-19.[8—14]



Triazavirin

The biological activity of nitro compounds of the azolotriazine series is usually attributed to the structural affinity for purine bases, due to which they can act as antimetabolites, exerting an antiviral effect.[3]

An important point in the early stages of molecular screening is the evaluation of the activity of compounds in experiments *in vitro* and *in vivo*. It is known that many drugs, especially nitro compounds, undergo redox transformations under *in vivo* conditions, which can affect their biological activity.[15,16] In view of the extensive theoretical base, wide research and instrumental capabilities, electrochemical methods are a convenient and reliable tool for the corresponding assessment. In the course of previous studies with the use of an integrated approach and combined methods for analysis, we proposed probable mechanisms of the electrochemical transformations of nitroazoloazines and showed their connection with the biological activity of a number of new antiviral compounds.[17,18]

The aims of the present study are the development of an efficient method for the synthesis of 4-hydroxy-3-nitro-1,4-dihydrotriazolo[5,1-*c*][1,2,4]triazines with an alkyl sulfanyl group in the triazole ring, the analysis of electrochemical transformations and antiviral activity of prepared compounds.

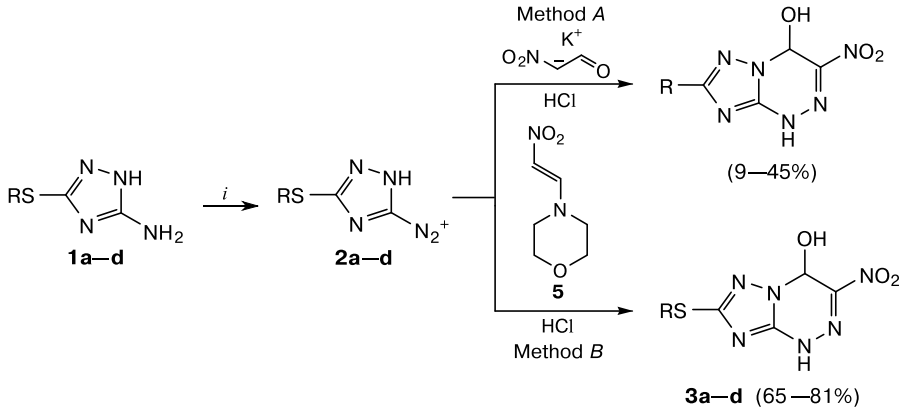
Results and Discussion

Synthesis of 4-hydroxy-3-nitro-1,4-dihydrotriazolo-[5,1-*c*][1,2,4]triazines. There are many methods for synthesis azolo[5,1-*c*][1,2,4]triazine structures and their close analogues.[19] At the same time, the search for new approaches to the synthesis of such compounds and the study of their properties are constantly underway.[20—22] It has previously been shown that promising building blocks for the synthesis of new nitroazolotriazines are nitrocarbonyl compounds, in particular, nitroacetaldehyde. A series of new 3-nitroazolo-1,2,4-triazines, including 4-hydroxy-7-methylsulfanyl-3-nitro-1,4-dihydrotriazolo[5,1-*c*][1,2,4]triazine were prepared using potassium salt of nitroacetaldehyde (Scheme 1, method *A*), however, the use of this nitro synthone is characterized by low yields.[23]

To synthesize target 4-hydroxy-3-nitro-1,4-dihydrotriazolo[5,1-*c*][1,2,4]triazines **3a—d** containing different alkyl sulfanyl substituents at position 7, we suggested to use of azo coupling of triazolyldiazonium salts **2a—d** directly with 1-nitro-2-morpholinoethylene **5**, which by its nature is a hidden form (crypto form) of nitroacetic aldehyde. Salts **2a—d** were synthesized from corresponding amino-1,2,4-triazoles **1a—d** (Scheme 1, method *B*).

By varying the reaction conditions, it is found that the highest yield of target 4-hydroxy-3-nitro-1,4-

Scheme 1



R = Me (**a**), Et (**b**), Pr^n (**c**), (**d**)

Reagents and conditions: *i*. $NaNO_2$, HCl, H_2O, −10 °C, 10 min; Method *A*: H_2O, 25 °C, 60 min; Method *B*: 1) MeCN, 0 °C, 60 min; 2) 25 °C, 12 h.

dihydrotriazolo[5,1-*c*][1,2,4]triazines **3a—d** (60—80%) can be achieved when the reaction of ethylene **5** (1 equiv.) with azolyldiazonium salts is carried out in acetonitrile in the presence of excess of hydrochloric acid. An additional advantage of the developed method is the absence of the need for chromatographic purification of target products **3a—d**, which is necessary when using method *A* (Scheme 1).

In ^{1}H NMR spectra of the synthesized compounds, there are three characteristic signals corresponding to the NH proton at δ 13.34—13.40, the proton at the C(4) atom of triazine ring at δ 6.90—6.91, and the hydroxyl proton at δ 8.08—8.13, as well as signals of alkyl sulfanyl groups. In ^{13}C NMR spectra, there are the characteristic signal at δ 72.6—72.8 (C(4)) and signals at δ 130—150 due to the C(3), C(7), and C(8a) atoms.

Electrochemical studies. Electrochemical transformations of a number of prepared 4-hydroxy-3-nitro-1,4-dihydrotriazolo[5,1-*c*][1,2,4]triazines were studied in water-organic buffer solutions by cyclic voltammetry and chronoamperometry.

According to the literature data,[18] the electrochemical transformations of various azoloazines are similar in general. As shown earlier, electrochemical activity of triazolotriazines is mainly determined by electroreduction of the nitro group.[14,20,21] Compounds **3a—d** have characteristic voltammograms (Fig. 1). In voltammograms of 4-hydroxy-3-nitro-1,4-dihydrotriazolo[5,1-*c*][1,2,4]triazines, there is a pronounced peak of the reduction of the nitro compound to the corresponding dimer at –0.5 V, as well as prominent anodic peaks at 0.2 and 1.1 V. Presumably, the anodic peak at $E = 0.2$ V in the voltammogram corresponds to the oxidation of the nitro compound to the corresponding hydroxylamine derivative, which agrees with literature data,[18] and the latter derivative is oxidized further to the nitroso compound at a potential of 1.1 V. It is found that the alkyl sulfanyl substituents in the structure of the studied molecules does not affect significantly on the magnitude and position of the peaks in the voltammograms.

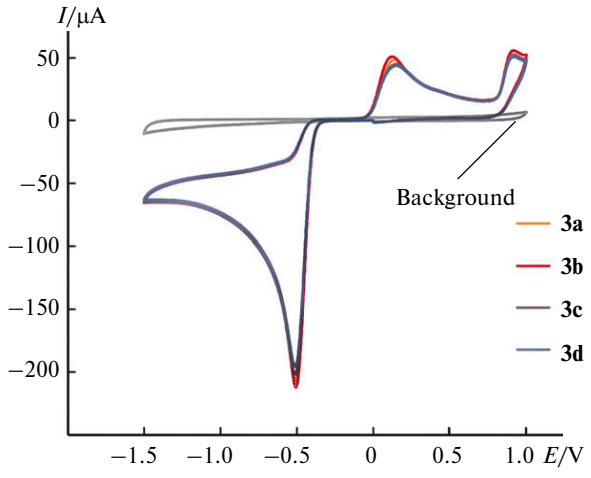


Fig. 1. Cyclic voltammograms of compounds **3a—d** ($C = 5$ mmol L^{-1}) recorded using a glassy carbon disk electrode in water-organic solutions consisted of the Britton—Robinson buffer (BRB) : DMSO (9 : 1), pH 7; potential sweep rate is 100 mV s^{-1}.

The effective number of electrons for each compound was calculated as the ratio of the peak current of the test substance to the peak current of one-electron reduction of potassium ferrocyanide under similar conditions. The found numbers of electrons n range from 2.76 to 2.93.

As can be seen, compounds **3a—d** have similar electrochemical behavior, therefore, further study was carried out with 4-hydroxy-3-nitro-7-propargylthio-1,4-dihydrotriazolo[5,1-*c*][1,2,4]triazine **3d** as a model compound.

The cathodic peak current linearly depends on the concentration of compound **3d** in the solution (Fig. 2).

Results of the study of electrotransformations of compound **3d** in a wide range of pH (Fig. 3) indicate the proton-dependent nature of the process. The dependency slope

$$E = (-0.0404 \pm 0.0005)\mathrm{pH} - (0.2289 \pm 0.0110)$$

shows that an equivalent number of protons is involved in the process (see Fig. 3, *a*). Note that the maximum electroreduction current was observed at pH 4, whereas in an alkaline medium, the peak current value significantly decreased (see Fig. 3, *b*).

The electrochemical activity of 4-hydroxy-3-nitro-1,4-dihydroazolo[5,1-*c*][1,2,4]triazines is apparently due to redox transformations of the nitro group and the subsequent formation of the corresponding dimers

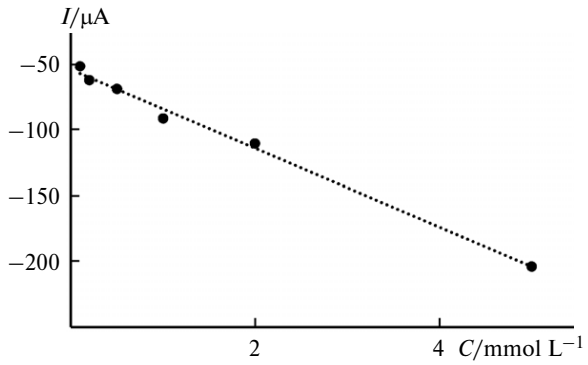


Fig. 2. Cathode peak current *vs.* the **3d** concentration in a BRB (pH 7) : DMSO (9 : 1) solution; $I = -29.959$ $C = 54.152$, $R^2 = 0.9936$; potential sweep rate is 100 mV s^{-1}.

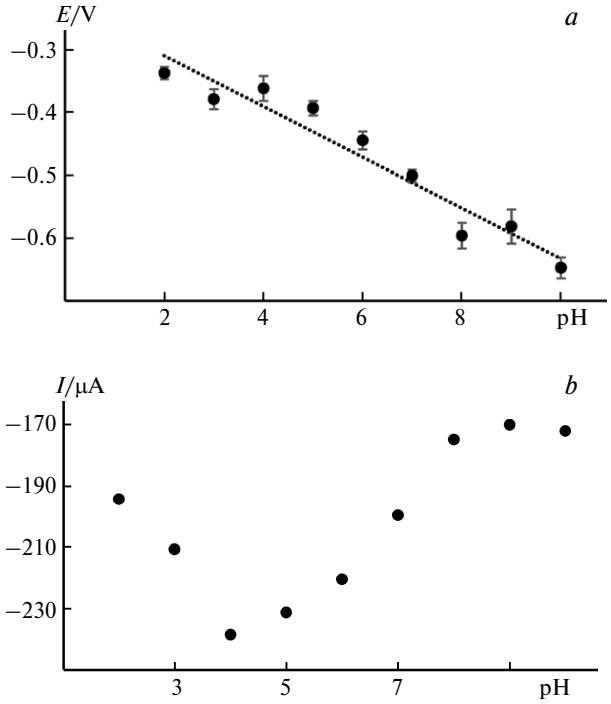
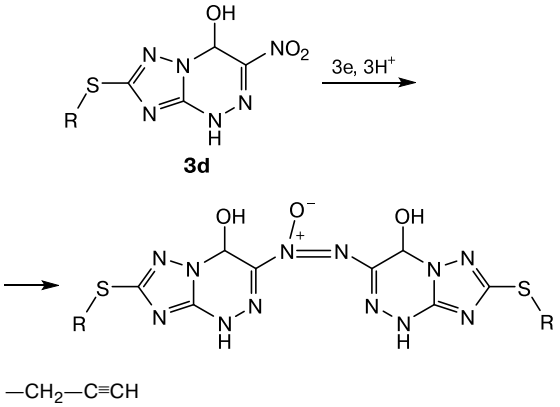


Fig. 3. Dependencies of the potential ($n = 3$, $P = 0.95$) (*a*) and current (*b*) of the electroreduction peak of compound **3d** on pH in a Britton—Robinson buffer : DMSO (9 : 1) solution, potential sweep rate is 100 mV s^{-1}, $C = 5$ mmol L^{-1}; $E = (-0.0404\pm0.0005)pH - (0.2289\pm0.0110)$.

(Scheme 2). It is established that the nature of the electrochemical transformations does not depend on the alkyl sulfanyl substituent in the triazole ring of the molecules. The obtained data are in agreement with literature data.[18,24,25]

Scheme 2



Taking into account the pronounced nature of redox transformations of compounds **3a—d**, it can be assumed that all studied molecules have biological activity.

Evaluation of toxicity and antiviral activity. The results of the evaluation of toxicity of compounds **3a—d** for the Vero cell culture and their antiviral activity against the Coxsackie B3 virus *in vitro* are collected in Table 1.

All compounds have relatively low toxicity, CC_{50} = = 345—785 μmol L^{-1}, and pronounced antiviral activity. Compound **3d** exhibits the highest activity ($IC_{50} = 2$ μmol L^{-1}), which in combination with rather low toxicity gives the highest selectivity of 307. Compounds **3b** and **3c** the same IC_{50} values (5 μmol L^{-1}). However, because of approximately twofold difference in toxicity, their selectivity is different: SI = 115 and 68, respectively.

The selectivity indexes obtained for the studied compounds far exceed the threshold value (SI = 10), which determines the prospects for using one or another compound as a potential antiviral agent.[26] It convincingly proves the high virus-inhibiting characteristics of the synthesized compounds.

To sum up, an efficient approach to the synthesis of new 4-hydroxy-3-nitro-1,4-dihydrotriazolo[5,1-*c*]-[1,2,4]triazines containing alkyl sulfanyl substituents in the triazole ring was proposed in this study. It is based on azo coupling of azolyldiazonium salts directly with nitromorpholinoethylene. Synthesized compounds **3a—d** exhibit pronounced redox properties due to the electroreduction of the nitro groups of the molecules and subsequent dimerization of the products of cathodic electrolysis. It is shown that the nature of alkyl sulfanyl substituents in triazolotriazines **3a—d** has no significant effect on the type of redox transformations in the series of the studied compounds.

The synthesized compounds have low toxicity ($IC_{50} \leqslant 14$ μmol L^{-1}) and pronounced antiviral activity against the Coxsakie virus *in vitro*. The highest activity (SI = 307) is shown by compound **3d**, which, in combination with its low toxicity, makes it possible to consider it as a promising object of further studies.

Table 1. Cytotoxicity and antiviral activity of compounds **3a—d** against the Coxsackie B3 virus in a Vero cell culture *in vitro*

Compound	CC_{50}	IC_{50}	SI
	μmol L^{-1}		
3a	785±42	14±3	55
3b	566±31	5±1	115
3c	345±22	5±1	68
3d	605±46	2±0.4	307
Ribavirin	>2130	56±8	38

Experimental

IR spectra were recorded on a Bruker Alpha spectrometer, ZnSe (ATR). ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Avance II spectrometer (400 and 100 MHz, respectively) in DMSO-d_6. The δ values in ^{1}H and ^{13}C spectra are given relative to $SiMe_4$ used as an internal standard. Elemental analysis was carried out on a Perkin Elmer 2400 Series II CHNS analyzer. Melting points were determined using a Staffordshire, ST15 0SA apparatus. The purity of prepared compounds was controlled by TLC on Silufol UV-254 plates.

Synthesis of 7-alkylsulfanyl-4-hydroxy-3-nitro-1,4-dihydro-1,2,4-triazolo[5,1-*c*][1,2,4]triazines 3a—d (general procedure). A solution of KNO_2 (0.936 g, 0.011 nol) in water (3 mL) was added in portions under stirring to a mixture of 3-amino-1,2,4-triazole (0.01 mol), water (5 mL), acetonitrile (5 mL), and concentrated HCl (10 mL, 0.12 mol) cooled down to −(7—10) °C. The reaction mixture was kept at this temperature for 10 min. Then, a solution of 1-nitro-2-morpholinoethylene (1.58 g, 0.01 mol) in acetonitrile (60 mL) was added to the mixture, and the resulting reaction mixture was kept at 0 °C for 1 h and, then, at room temperature for 12 h. The precipitate formed was filtered and washed with a cooled acetonitrile : water (1 : 1) mixture. The wet precipitate was recrystallized from water, filtered off, and dried in air.

4-Hydroxy-7-methylthio-3-nitro-1,4-dihydro-1,2,4-triazolo[5,1-*c*][1,2,4]triazine (3a). Yield 1.75 g (75%), an orange powder, m.p. 194—196 °C (dec.). Found (%): C, 25.96; H, 2.44; N, 36.45. $C_5H_6N_6O_3S$. Calculated (%): C, 26.09; H, 2.63; N, 36.51. IR, ν/cm^{-1}: 678, 700, 723, 753, 824, 904, 1066, 1113, 1181, 1240, 1276, 1328, 1389, 1536, 2804, 2862. ^{1}H NMR, δ: 13.22 (s, 1 H, NH); 7.99 (d, 1 H, OH, J = 7.6 Hz); 6.89 (d, 1 H, CH, J = 7.6 Hz); 2.56 (s, 3 H, SCH_3). ^{13}C NMR, δ: 13.53, 72.29, 142.44, 146.71, 161.31.

7-Ethylthio-4-hydroxy-3-nitro-1,4-dihydro-1,2,4-triazolo[5,1-*c*][1,2,4]triazine (3b). Yield 1.80 g (74%), bright yellow powder, m.p. 156—160 °C. Found (%): C, 29.40; H, 3.59; N, 34.81. $C_6H_8N_6O_3S$. Calculated (%): C, 29.51; H, 3.30; N, 34.41. IR, ν/cm^{-1}: 646, 866, 932, 1015, 1056, 1189, 1330, 1776, 1789, 3211, 3222, 3235, 3243. ^{1}H NMR, δ: 13.40 (s, 1 H, NH); 8.13 (s, 1 H, OH); 6.91 (s, 1 H, CH); 3.10 (q, 2 H, CH_2, J = 7.3 Hz); 1.33 (t, 1 H, CH, J = 7.3 Hz). ^{13}C NMR, δ: 160.90, 147.23, 142.92, 72.80, 25.66, 15.50.

4-Hydroxy-3-nitro-7-propylthio-1,4-dihydro-1,2,4-triazolo[5,1-*c*][1,2,4]triazine (3c). Yield 2.10 g (81%), bright yellow powder, m.p. 163—164 °C. Found (%): C, 32.40; H, 3.69; N, 32.81. $C_7H_{10}N_6O_3S$. Calculated (%): C, 32.41; H, 3.90; N, 32.54. IR, ν/cm^{-1}: 598, 1302, 1514, 1792, 3245. ^{1}H NMR, δ: 13.39 (s, 1 H, NH); 8.13 (d, 1 H, OH, J = 7.0 Hz); 6.91 (d, 1 H, CH, J = 7.0 Hz); 3.07 (t, 2 H, CH_2, J = 7.0 Hz); 1.7 (m, 2 H, CH_2, J = 7.3 Hz); 0.97 (t, 3 H, CH_3, J = 7.3 Hz). ^{13}C NMR, δ: 160.65, 146.65, 142.49, 72.36, 32.79, 22.57, 13.05.

4-Hydroxy-3-nitro-7-propargylthio-1,4-dihydro-1,2,4-triazolo[5,1-*c*][1,2,4]triazine (3d). Yield 1.66 g (65%), light yellow powder, m.p. 210—212 °C. Found (%): C, 32.88; H, 2.44; N, 32.82. $C_7H_6N_6O_3S$. Calculated (%): C, 33.07; H, 2.38; N, 33.06. IR, ν/cm^{-1}: 537, 1292, 1514, 1692, 3295. ^{1}H NMR, δ: 13.34 (s, 1 H, NH); 8.08 (s, 1 H, OH); 6.90 (s, 1 H, CH); 4.14 (d, 2 H, CH_2, J = 2.6 Hz); 1.47 (t, 1 H, CH, J = 2.6 Hz). ^{13}C NMR, δ: 159.29, 146.88, 142.62, 80.19, 73.90, 72.57, 19.38.

Electrochemical studies. Electrochemical measurements were performed on a μAutolab Type III potentiostat (Metrohm Autolab, Netherlands) by cyclic voltammetry (CV). All measurements were carried out in a glass three-electrode cell (working solution volume was 10 mL). A glassy carbon disk was used as the working electrode (working area was 7 mm^2). Measurements were carried out relative to a silver chloride electrode Ag/Ag^+/KCl at the potential sweep rate of 100 mV s^{-1}. Cyclig voltammograms were recorded for water-organic solutions of compounds (5 mmol L^{-1}) in mixtures of DMSO : Britton—Robinson buffer (BRB) (1 : 9). The Britton—Robinson buffer solution was prepared by mixing orthoposphoric (0.04 mol L^{-1}), acetic (0.04 mol L^{-1}), and boric (0.04 mol L^{-1}) acids and bringing to the required pH 2—10 with sodium hydroxide.

Biological studies. Experiments were performed on the Vero cell line (ATCC CCL-81), which was seeded into wells of 96-well cell culture plates and incubated until a 90% monolayer was formed. In experiments on antiviral activity, the Coxsackie B3 virus (Nancy strain) obtained from the collection of viral cultures of the Pasteur Institute of Epidemiology and Microbiology was used.

Evaluation of cytotoxic properties. To assess toxicity, a series of two-fold dilutions were prepared from the studied compounds at concentrations from 300 to 4 μg mL^{-1} on Eagle's MEM supporting medium. Cells were seeded into wells of 96-well cell culture plates (in the amount of 10000 per well) and incubated until a 90% monolayer was formed. Dilutions were introduced in the wells of cell culture plates and incubated for 72 h at 36 °C in an atmosphere of 5% CO_2. Then the microtetrazolium (MTT) test was performed on the 96-well cell culture plates.[27] Cells were washed twice with a physiological saline (0.9% NaCl), and a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in Eagle's MEM supporting medium in a concentration of 0.5 μg mL^{-1} was added in an amount of 100 μL per well. The cell culture plates were incubated for 1 h at 36 °C. After incubation with the dye, the culture medium was removed, and 0.1 mL of DMSO was added to each well to dissolve the precipitate. Measurements of optical density were carried out on a ThermoMultiskan FC spectrophotometer (ThermoFisherScientific, USA) at 540 nm. The intensity of MTT staining of cell samples

from control wells was taken as 100%. The CC_{50} values, which are compound concentrations leading to the death of 50% of the cells in culture, were calculated based on the obtained data.

Evaluation of antiviral activity *in vitro*. The antiviral activity of the compounds was evaluated by the test for reducing the degree of virus-specific cytopathic action. The compounds under study at concentrations from 3.7 to 300 μg mL^{-1} were introduced in wells of a cell culture plate, the cell culture plates were incubated for 1 h at 36 °C in an atmosphere of 5% CO_2, then, the cells were infected with the virus at a dose of 0.01 $TCID_{50}$ (50% of tissue cytotoxic doses) per cell. Infected cells were incubated for 72 h at 36 °C under the 5% CO_2 atmosphere, and the cell survival analysis using the MTT test was performed as described above. The 50% inhibiting concentration, *i.e.*, the concentration, which reduced the degree of viral destruction of cells by 50%, was calculated for each compound from the data obtained. The selectivity index (SI) representing the ratio of CC_{50} to IC_{50} was then calculated for each compound.

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No human or animal subjects were used in this research.

The authors declare no competing interests.

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