

SEARCH FOR NEW DRUGS

SYNTHESIS OF C5-C6 DERIVATIVES OF 1,3-DIMETHYL-5-FLUOROURACIL AND 5-FLUOROURACIL. SCREENING FOR ANTIVIRAL ACTIVITY

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Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 53, No. 2, pp. 16 – 20, February, 2019.

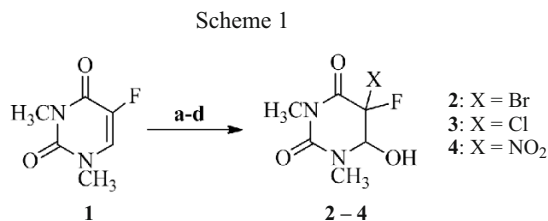
Original article submitted September 26, 2017

5-Chloro-5-fluoro-6-alkoxy-pyrimidines were synthesized via Cl₂ chlorination of 5-fluorouracil and 1,3-dimethyl-5-fluorouracil in various alcohols and were screened for antiviral activity. High antiviral activity was found for 5-chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil against RS virus and human flu viruses A/H3N2 and A/H1N1pdm09.

Keywords: 5-fluorouracil, 5-chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil, antiviral activity.

The development of simple and effective methods for direct introduction of functional groups into a pyrimidine ring is an important advance in the chemical modification of heterocyclic bases. Several uracil derivatives modified at the C5=C6 double bond possessed broad spectra of high pharmacological activity [1-6]. 5-Halouracil derivatives represent the most interesting series of medicines because they are produced *in vivo* by various inflammatory processes that can lead to mutations caused by damage to nucleic acids and subsequent oncological diseases [7-8]. It seemed advantageous to study the antiviral activity of the synthesized compounds against various strains of influenza and respiratory-syncytial virus (RSV) because seasonal respiratory infections are still the most common viral illnesses.

Previously developed methods for oxidative halogenation by KHal/H₂O₂—H₂SO₄ (20%) of 1,3-dimethyl-5-fluorouracil (**1**) were used to produce 1,3-dimethyl-5-bromo-5-fluoro- (**2**) and 1,3-dimethyl-5-chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil (**3**) in 85 and 40% yields, respectively. Chlorination of **1** in conc. HCl in the presence of H₂O₂ formed **3** in 64% yield. Nitration of **1** gave 1,3-dimethyl-5-fluoro-6-hydroxy-5-nitro-5,6-dihydrouracil (**4**) in 90% yield (Scheme 1).



Reagents and conditions: a. KBr (2 eq) — 33 % H₂O₂ (3 eq), 20 % H₂SO₄, 5 h, **2** (85 %); b. KCl (2 eq) — 33 % H₂O₂ (3 eq), 20 % H₂SO₄, 5 h, **3** (40 %); c. 37 % HCl (6 eq) — 33 % H₂O₂ (8 eq), 5 h, **3** (64 %); d. HNO₃—H₂SO₄, 0 – 10 °C, 4 h, **4** (90 %).

Theoretical calculations of the equilibrium geometries of **2-4** in the B3LYP/6-311+G(d,p)//B3LYP/6-311G(d,p)+IEFPCM approximation allowed the steric orientations of the F and OH substituents in the pyrimidinedione ring to be determined. Table 1 shows that the thermodynamically most stable isomer for all compounds **2-4** had an equatorial F atom and axial hydroxyl group because of an anomeric effect [3]. The populations of isomers were 99, 97, and 92% for X = Br, Cl, and NO₂, respectively.

5-Chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil exhibited pronounced antiviral activity against all studied flu subtypes and RSV, decreasing the virus infectious titer by 1.9 – 2.2 log-ID₅₀ units at a concentration of 0.137 mM. The antiviral activity of 5-chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil was greatest against RSV and human flu viruses A/H3N2 and A/H1N1pdm09. However, it was much less ac-

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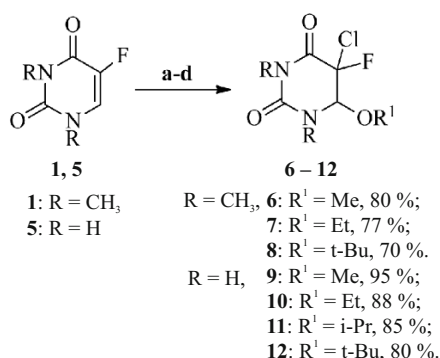
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tive against avian flu virus A/H5N1 and influenza type B virus according to both the EC_{50} value and maximum reduction of virus infectious titer in the experimental concentration range (Table 2). Also, the mean cytotoxic dose (CTD_{50}) of the compound was (2.05 ± 0.09) mM. However, the *N,N*-dimethyl analog of 5-chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil turned out to be inactive against all studied viruses up to the maximum tested concentration (0.475 mM). The activities of the reference drugs were $EC_{50} = 0.004$ mM and $SI > 100$ for oseltamivir carboxylate against A/California/07/09; $EC_{50} = 0.011$ mM and $SI = 5.75$ for arbidol against A/Perth/16/09; and $EC_{50} = 0.019$ mM and $SI = 3.25$ for arbidol against RSV/4317. Based on the results, the synthesis of 5-chloro-5-fluoro-substituted dihydrouracil derivatives with 6-OR groups, where R is a substituent different from H, seemed attractive.

Chlorination of 1,3-dimethyl-5-fluorouracil (**1**) and 5-fluorouracil (**5**) by Cl_2 in anhydrous MeOH, EtOH, *i*-PrOH, and *t*-BuOH formed a series of C-6 alkoxy derivatives (**6**–**12**) (Scheme 2). Chlorination of **1** by Cl_2 in *i*-PrOH gave a mixture of products consisting of 1,3-dimethyl-5-chloro-5-fluoro-6-isopropoxy-5,6-dihydrouracil and side products that could not be separated.

Scheme 2



Reagents and conditions: a. Cl_2 , MeOH, 2 h; b. Cl_2 , EtOH c. Cl_2 , *i*-PrOH, 2 h; d. Cl_2 , *t*-BuOH, 2 h.

Screening *in vitro* for cytotoxicity and antiviral activity of **9** and **11** against virus A(H1N1)pdm09 did not find antiviral activity.

EXPERIMENTAL CHEMICAL PART

PMR and ^{13}C NMR spectra were recorded on a Bruker Avance-III 500-MHz pulsed spectrometer at operating frequency 500.13 MHz (1H) and 125.76 MHz (^{13}C). Melting points were determined in glass capillaries. Quantum-chemical calculations used Gaussian-09, Revision C.1 software [9]. Geometries of all conformers of **2**–**4** were fully optimized and force constants were calculated using electron-density hybrid functional B3LYP [10, 11] and basis set 6-311G(d, p) [12]. Total energies were found from a single calculation for the optimized states using diffuse basis set

6-311+G(d, p). The resulting structures corresponded to minima on the potential-energy surface of the studied compounds according to the lack of negative elements in the diagonalized Hessian matrix. Free energies (G^0) of compounds and transition states were calculated using the equation:

$$G^0 = E_{\text{tot}} + ZPE + TC_G$$

where E_{tot} is the total energy of the compound; ZPE , energy of null vibrations; and TC_G , thermal correction to the free energy calculated at 298 K.

Solvent ($CHCl_3$) effects on the populations of conformational states were taken into account using an IEFPCM continuum model [13, 14].

1,3-Dimethyl-5-bromo-5-fluoro-6-hydroxy-5,6-dihydrouracil (2). A mixture of 1,3-dimethyl-5-fluorouracil (0.20 g, 1.27 mmol) and KBr (0.30 g, 2.54 mmol) in H_2SO_4 (20%, 2.00 mL) was treated dropwise with H_2O_2 (33%, 0.51 mL, 5.08 mmol), stirred for 5 h at room temperature, diluted with H_2O , and extracted by $CHCl_3$. The combined extracts were washed with H_2O , dried over Na_2SO_4 , evaporated, and crystallized from Me_2CO to give a yellow amorphous powder, yield 0.27 g (85%). PMR spectrum ($DMSO-d_6$), δ , ppm: 3.05 (3H, c, H-3); 3.11 (3H, c, H-1); 3.62 (1H, br.s, OH); 5.04 (1H, d, H-6, $J = 1.1$ Hz). ^{13}C NMR spectrum ($DMSO-d_6$), δ , ppm: 28.15 (CH_3N-3); 34.84 (CH_3N-1); 84.28 (d, C-6, $J = 27.7$ Hz); 90.13 (d, C-5, $J = 265.4$ Hz); 151.14 (C-2); 163.36 (d, C-4, $J = 25.2$ Hz).

1,3-Dimethyl-5-chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil (3).

Method 1. A mixture of 1,3-dimethyl-5-fluorouracil (0.20 g, 1.27 mmol) and KCl (0.19 g, 2.54 mmol) in H_2SO_4 (20%, 2.00 mL) was treated dropwise with H_2O_2 (33%, 0.51 mL, 5.08 mmol), stirred for 5 h at room temperature, diluted with H_2O , and extracted by $CHCl_3$. The combined extracts were washed with H_2O , dried over Na_2SO_4 , evaporated, and crystallized from Me_2CO to give a white amorphous powder, yield 0.11 g (40%).

Method 2. 1,3-Dimethyl-5-fluorouracil (0.20 g, 1.27 mmol) was stirred in HCl (37%, 0.23 mL, 7.62 mmol, ρ 1.185 g/mL), treated dropwise with H_2O_2 (33%, 1.02 mL, 10.16 mmol), stirred for 5 h at room temperature, diluted with H_2O , and extracted by $CHCl_3$. The combined extracts were washed with H_2O , dried over Na_2SO_4 , evaporated, and crystallized from Me_2CO to give a white amorphous powder, yield 0.17 g (64%).

PMR spectrum ($DMSO-d_6$), δ , ppm: 3.11 (3H, c, H-3); 3.16 (3H, c, H-1); 3.50 (1H, br.s., OH); 5.02 (1H, d, H-6, $J = 1.8$ Hz). ^{13}C NMR spectrum ($DMSO-d_6$), δ , ppm: 28.38 (CH_3N-3); 35.05 (CH_3N-1); 83.66 (d, C-6, $J = 27.7$ Hz); 96.45 (d, C-5, $J = 254.0$ Hz); 151.18 (C-2); 162.61 (d, C-4, $J = 27.7$ Hz).

1,3-Dimethyl-5-fluoro-6-hydroxy-5-nitro-5,6-dihydrouracil (4). 1,3-Dimethyl-5-fluorouracil (0.20 g, 1.27 mmol)

was dissolved gradually with stirring in conc. H_2SO_4 (0.60 mL, $\rho = 1.835 \text{ g/mL}$), cooled to 0°C after the starting compound dissolved completely, treated dropwise with HNO_3 (0.40 mL, $\rho = 1.395 \text{ g/mL}$), held at $0 - 10^\circ\text{C}$ for 4 h, diluted with H_2O , and extracted with CHCl_3 . The combined extracts were washed with H_2O , dried over Na_2SO_4 , evaporated, and crystallized from Me_2CO to give a yellow amorphous powder, yield 0.25 g (90%).

PMR spectrum (DMSO-d_6), δ , ppm: 2.98 (3H, c, H-3); 3.22 (3H, c, H-1); 3.68 (1H, br.s., OH); 5.65 (1H, d, H-6, $J = 3.3 \text{ Hz}$). ^{13}C NMR spectrum (DMSO-d_6), δ , ppm: 28.40 ($\text{CH}_3\text{N-3}$); 34.45 ($\text{CH}_3\text{N-1}$); 79.68 (d, C-6, $J = 25.2 \text{ Hz}$); 106.50 (d, C-5, $J = 246.5 \text{ Hz}$); 151.37 (C-2); 157.78 (d, C-4, $J = 25.2 \text{ Hz}$).

Syntheses of 1,3-dimethyl-5-chloro-5-fluoro-6-alkoxy-5,6-dihydrouracils (general method). A solution of 1,3-dimethyl-5-fluorouracil (0.20 g, 1.27 mmol) in anhydrous alcohol (5.00 mL, MeOH, EtOH, *i*-PrOH, *t*-BuOH) was purged with Cl_2 for 30 min, stirred at room temperature for 2 h, evaporated to dryness in vacuo, and chromatographed over SiO_2 using CHCl_3 eluent.

1,3-Dimethyl-5-chloro-5-fluoro-6-methoxy-5,6-dihydrouracil (6). Yellow oil, yield 0.23 g (80%). PMR spectrum (DMSO-d_6), δ , ppm: 3.12 (6H, c, $\text{CH}_3\text{N-3}$, $\text{CH}_3\text{N-1}$); 3.49 (3H, s, H-7); 4.68 (1H, br.s., H-6). ^{13}C NMR spectrum (DMSO-d_6), δ , ppm: 28.22 ($\text{CH}_3\text{N-3}$); 36.26 ($\text{CH}_3\text{N-1}$); 58.87 (d, C-7, $J = 3.8 \text{ Hz}$); 91.12 (d, C-6, $J = 25.2 \text{ Hz}$); 96.53 (d, C-5, $J = 257.8 \text{ Hz}$); 150.70 (C-2); 161.89 (d, C-4, $J = 26.4 \text{ Hz}$).

1,3-Dimethyl-5-chloro-5-fluoro-6-ethoxy-5,6-dihydrouracil (7). Yellow oil, yield 0.23 g (77%). PMR spectrum (DMSO-d_6), δ , ppm: 1.17 (3 H, m, H-8); 3.10 (3H, c, $\text{CH}_3\text{N-3}$); 3.15 (3H, s, $\text{CH}_3\text{N-1}$); 3.68 (3H, m, H-7); 5.01 (1H, d, H-6, $J = 5.2 \text{ Hz}$). ^{13}C NMR spectrum (DMSO-d_6), δ , ppm: 15.00 (d, C-8, $J = 7.5 \text{ Hz}$); 28.20 ($\text{CH}_3\text{N-3}$); 35.83 ($\text{CH}_3\text{N-1}$); 67.48 (d, C-7, $J = 2.5 \text{ Hz}$); 89.92 (d, C-6, $J = 25.2 \text{ Hz}$); 96.49 (d, C-5, $J = 259.1 \text{ Hz}$); 150.81 (C-2); 161.95 (d, C-4, $J = 27.7 \text{ Hz}$).

1,3-Dimethyl-5-chloro-5-fluoro-6-*t*-butoxy-5,6-dihydrouracil (8). Yellow oil, mp $102 - 105^\circ\text{C}$ (CHCl_3), yield 0.24 g (70%). PMR spectrum (DMSO-d_6), δ , ppm: 1.02 (9H, s, H-8, H-9, H-10); 3.20 (3H, s, $\text{CH}_3\text{N-3}$); 3.40 (3H, s, $\text{CH}_3\text{N-1}$); 4.57 (1H, d, H-6, $J = 1.2 \text{ Hz}$). ^{13}C NMR spectrum (DMSO-d_6), δ , ppm: 28.22 ($\text{CH}_3\text{N-3}$); 28.60 (C-8, C-9, C-10); 35.53 ($\text{CH}_3\text{N-1}$); 52.14 (C-7); 84.53 (d, C-6, $J = 26.4 \text{ Hz}$); 96.25 (d, C-5, $J = 259.1 \text{ Hz}$); 151.50 (C-2); 162.29 (d, C-4, $J = 26.4 \text{ Hz}$).

Syntheses of 5-chloro-5-fluoro-6-alkoxy-5,6-dihydrouracils (general method). A solution of 5-fluorouracil (0.20 g, 1.54 mmol) in anhydrous alcohol (5.00 mL, MeOH, EtOH, *i*-PrOH, *t*-BuOH) was purged with Cl_2 for 30 min, stirred at room temperature for 2 h, and evaporated to dryness in vacuo. The solid was dissolved in H_2O and extracted by Et_2O ($3 \times 15 \text{ mL}$). The combined extracts were washed with distilled H_2O ($1 \times 15 \text{ mL}$), dried over Na_2SO_4 , evaporated, and recrystallized from Et_2O .

5-Chloro-5-fluoro-6-methoxy-5,6-dihydrouracil (9). White crystals, mp $201 - 203^\circ\text{C}$ (Et_2O), yield 0.31 g (95%). PMR spectrum (DMSO-d_6), δ , ppm: 3.35 (3H, c, H-7); 4.98

TABLE 1. Absolute Free Energies G_{298}^0 (Hartree) and Relative Free Energies ΔG_{298}^0 (kJ/mol) of Isomers of **2** – **4** and Conformer Populations (%)

Compound	Substituent orientation		G_{298}^0	ΔG_{298}^0	Conformer population
	F	OH			
2	<i>a</i>	<i>a</i>	-3242.851872	14.6	0
	<i>a</i>	<i>e</i>	-3242.848826	22.6	0
	<i>e</i>	<i>a</i>	-3242.857429	0.0	99
	<i>e</i>	<i>e</i>	-3242.852283	13.5	0
3	<i>a</i>	<i>a</i>	-1128.933567	9.6	2
	<i>a</i>	<i>e</i>	-1128.930001	19.0	0
	<i>e</i>	<i>a</i>	-1128.937238	0.0	97
	<i>e</i>	<i>e</i>	-1128.932257	13.1	0
4	<i>a</i>	<i>a</i>	-873.863406	8.0	4
	<i>a</i>	<i>e</i>	-873.863699	7.3	5
	<i>e</i>	<i>a</i>	-873.866466	0.0	92
	<i>e</i>	<i>e</i>	-873.859353	18.7	0

* Calculations in B3LYP/6-311+G(d,p)//B3LYP/6-311G(d,p) approximation considering solvent (CHCl_3 used to record NMR spectra) effects in terms of the IEFPCM polarized continuum model.

(1H, d, H-6, J = 4.9 Hz); 9.24 (1H, c, H-1); 11.19 (1H, c, H-3). ¹³C NMR spectrum (DMSO-d₆), δ, ppm: 56.31 (C-7); 83.90 (d, C-6, J = 25.2 Hz); 96.66 (d, C-5, J = 257.8 Hz); 150.91 (C-2); 162.83 (d, C-4, J = 27.7 Hz).

5-Chloro-5-fluoro-6-ethoxy-5,6-dihydrouracil (10).

White crystals, mp 180 – 182°C (Et₂O), yield 0.28 g (88%). PMR spectrum (DMSO-d₆), δ, ppm: 1.05 (3H, t, H-8, J = 7 Hz); 3.64 (2H, m, H-7); 5.03 (1H, d, H-6, J = 4.7 Hz); 9.15 (1H, c, H-1); 11.15 (1H, c, H-3). ¹³C NMR spectrum (DMSO-d₆), δ, ppm: 15.27 (C-8); 64.62 (C-7); 82.64 (d, C-6, J = 23.9 Hz); 96.66 (d, C-5, J = 259.1 Hz); 150.99 (C-2); 162.88 (d, C-4, J = 26.4 Hz).

5-Chloro-5-fluoro-6-isopropoxy-5,6-dihydrouracil (11).

White crystals, mp 195 – 197°C (Et₂O), yield 0.29 g (85%). PMR spectrum (DMSO-d₆), δ, ppm: 1.09 and 1.10 (6H, d, H-8, H-9, J = 6.2); 3.91 (1H, m, H-7); 5.10 (1H, d, H-6, J = 0.7 Hz); 9.10 (1H, c, H-1); 11.10 (1H, c, H-3). ¹³C NMR spectrum (DMSO-d₆), δ, ppm: 21.77 and 22.94 (C-8, C-9); 71.21 (C-7); 81.04 (d, C-6, J = 25.2 Hz); 96.39 (d, C-5, J = 259.1 Hz); 150.83 (C-2); 162.68 (d, C-4, J = 27.7 Hz).

5-Chloro-5-fluoro-6-*t*-butoxy-5,6-dihydrouracil (12).

White crystals, mp 198 – 200°C (Et₂O), yield 0.31 g (80%). PMR spectrum (DMSO-d₆), δ, ppm: 1.02 (9H, s, H-8, H-9, H-10); 5.02 (1H, d, H-6, J = 1.0 Hz); 9.00 (1H, c, H-1); 11.00 (1H, c, H-3). ¹³C NMR spectrum (DMSO-d₆), δ, ppm: 28.45 (C-8, C-9, C-10); 77.14 (C-7); 77.79 (d, C-6, J = 25.2 Hz); 96.66 (d, C-5, J = 257.8 Hz); 151.22 (C-2); 163.01 (d, C-4, J = 27.7 Hz).

EXPERIMENTAL BIOLOGICAL PART

All studies complied with the *Handbook for Preclinical Drug Trials* [17] using primary culture of MDCK canine kidney cells. Preliminary screening for antiviral activity used reference strain A/Perth/16/09 A(H3N2) with adequate hemagglutinating (1:128) and infectious activity (7.0 log

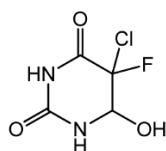
ID₅₀/50 μL) [15]. Compounds exhibiting pronounced antiviral activity against influenza strain A/H3N2 were tested against reference strains of other flu subtypes, i.e., pandemic group 2009, strain A/California/08/09(H1N1)pdm09; avian flu virus A(H5N1), A/NIBRG/14; influenza B virus, strain B/Brisbane/60/08; and a standard RSV strain. All used strains were authentic standard strains recommended by WHO Collaborating Centres for Influenza (CDC, Atlanta, USA and F. Crick Inst., Crick Worldwide Influenza Centre, London, UK) as candidates for vaccine strains. All strains except A/NIBRG14 were obtained from CDC (USA) and are preserved in the Virus Collection of the Research Institute of Influenza. The last was a highly pathogenic strain of avian flu with a remote pathogenic site (site of proteolytic cleavage of hemagglutinin) and was obtained from the National Institute for Biological Standards and Control (NIBSC, London).

Cytotoxicities of the compounds were checked as follows. A weighed portion of each compound (5 mg) was placed into a sterile 5-mL tube. The 5-chloro-5-fluoro-6-alkoxy-pyrimidines were diluted in DMSO to a concentration of 20 mg/mL to produce stock solutions. Immediately before an experiment, the stock solution was diluted in incubation medium by 100× so that the final DMSO concentration was <1% (maximum allowed concentration of DMSO without affecting cell vitality and metabolism). In this manner, the maximum working concentration of the tested compounds was 200 μg/mL. Tests were performed in triplicate for each concentration. Medium not containing serum was rinsed away. The compounds were added. The plates were incubated for 72 h at 37°C with 5% CO₂.

The criterion for cytotoxicity *in vitro* was the reduction by cells in culture of tetrazolium dye MTT (thiazolyl blue, Sigma, USA), the extent of which indicated the cell vitality level for reducing the dye by mitochondrial and partially cytoplasmic dehydrogenases [16].

Results for MTT reduction were measured on a Variokan microplate reader (Thermo Fisher) at 550 nm. The mean

TABLE 2. Antiviral Activity of 5-Chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil



Virus	Maximum decrease of infectious virus titer (Δlog ID ₅₀) at conc. 50 μg/mL (0.273 mM)	EC ₅₀ , mM	SI = CTD ₅₀ /EC ₅₀
A/Perth/16/09 (H3N2)	1.90 ± 0.24	0.228 ± 0.019	9.0
A/California/07/09 (H1N1)pdm09	0.95 ± 0.14	0.186 ± 0.017	11.0
A/NIBRG/14 (H5N1)	0.80 ± 0.17	0.433 ± 0.053	4.7
B/Brisbane/60/08	0.68 ± 0.11	0.391 ± 0.035	5.3
RS virus RSV/4317	2.26 ± 0.21	0.124 ± 0.016	16.5

cytotoxic dose (CTD₅₀) was determined by linear regression of the photometric results using the program Excel 2010. All calculated data gave regressions with $R^2 > 0.9$.

Antiviral activity of the 5-chloro-5-fluoro-6-alkoxy-pyrimidines against MDCK cells was assessed using double dilutions of each compound starting with the maximum nontoxic concentration, i.e., the maximum concentration at which 100% of the monolayer cells survived.

Plates with one-day monolayers of MDCK cells were rinsed twice with medium not containing serum. Then, compounds (50 μ L) were placed into the corresponding plate wells. Control wells were filled with the same volume of growth medium. Plates were incubated for 60 min at 37°C with 5% CO₂. Next, virus in the range 10⁵ – 10⁻² TID₅₀ was added. Each compound concentration was tested in triplicate for each virus dilution. Control wells were filled with the same volume of growth medium. Plates were incubated for 48 h at 37°C with 5% CO₂. Virus reproduction was assessed using hemagglutination (HA) and was expressed as the reduction of HA titer as compared to a control (untreated virus in 10-fold dilutions), log ID₅₀/50 μ L. Cytopathogenic activity of the viruses was quantified also using the MTT assay and was expressed as Δ log ID₅₀/50 μ L as compared with a virus control [16, 17]. The important quantitative criteria for the antiviral activity were EC₅₀, the mean effective concentration, i.e., the concentration causing 50% protection vs. a virus control (infected cells without tested compound), and the selectivity index (SI) (pharmaceutical or therapeutic index), $SI = CTD_{50}/ED_{50}$. The reference drug was oseltamivir carboxylate (Hoffmann-LaRoche, Switzerland) for virus A/California/07/09 (H1N1)pdm09 and arbidol (Farmstandart, Russia) for viruses A/Perth/16/09 (H3N2) and RSV/4317. Table 2 presents the mean EC₅₀ and SI values obtained from HA and MTT assays.

ACKNOWLEDGMENTS

The work was performed on State Task Topic “Synthesis of biologically active heterocyclic and diterpenoid compounds” No. AAAA-A17 – 117011910025. Spectral and quantum-chemical parts used equipment at the Khimiya CUC, UfIC, RAS.

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