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Antiviral properties of synthetic histidine derivatives containing membranotropic volumetrical carbocycles in their molecule against SARS-CoV-2 virus *in vitro*

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Introduction. Currently, low molecular-weight compounds are being developed as potential inhibitors of CoVs replication, targeting various stages of the replication cycle, such as major protease inhibitors and nucleoside analogs. Viroporins can be alternative protein targets.
The **aim** of this study is to identify antiviral properties of histidine derivatives with cage substituents in relation to pandemic strain SARS-CoV-2 *in vitro*.
Materials and methods. Combination of histidine with aminoadamantane and boron cluster anion $[B_{10}H_{10}]^{2-}$ (compounds **I–IV**) was carried out by classical peptide synthesis. Compound were identified by modern physicochemical methods. Antiviral properties were studied *in vitro* on a monolayer of Vero E6 cells infected with SARS-CoV-2 (alpha strain) with simultaneous administration of compounds and virus.
Results. Derivatives of amino acid histidine with carbocycles and boron cluster were synthesized and their antiviral activity against SARS-CoV-2 was studied *in vitro*. Histidine derivatives with carbocycles and $[B_{10}H_{10}]^{2-}$ have the ability to suppress virus replication. The solubility of substances in aqueous media can be increased due to formation of hydrochloride or sodium salt.
Discussion. 2HCl*H-His-Rim (**I**) showed some effect of suppressing replication of SARS-CoV-2 at a viral load of 100 doses and concentration 31.2 μg/ml. This is explained by the weakly basic properties of compound **I**.
Conclusion. The presented synthetic compounds showed moderate antiviral activity against SARS-CoV-2. The obtained compounds can be used as model structures for creating new direct-acting drugs against modern strains of coronaviruses.

Keywords: *adamantane derivatives; histidine amino acid; antiviral activity; SARS-CoV-2; boron cluster anions*

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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
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Противовирусные свойства синтетических производных гистидина, содержащих в своей молекуле мембранотропные объёмные карбоциклы, в отношении вируса SARS-CoV-2 *in vitro*

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Введение. В настоящее время разрабатывается целый ряд низкомолекулярных соединений в качестве потенциальных ингибиторов репликации CoVs, направленных на различные этапы репликационного цикла, такие как ингибиторы основной протеазы и аналоги нуклеозидов. Альтернативной белковой мишенью могут выступать виропорины.
Цель исследования – выявление противовирусных свойств производных гистидина с каркасными заместителями в отношении пандемического штамма коронавируса SARS-CoV-2 *in vitro*.
Материалы и методы. Получение соединения гистидина с аминоадамантаном и декагидро-*клозо*-декаборатным анионом $[B_{10}H_{10}]^{2-}$ проведено методами классического пептидного синтеза. Структура соединения подтверждена современными физико-химическими методами. Противовирусные свойства синтетических соединений изучены *in vitro* на монослое клеток Vero E6, инфицированных SARS-CoV-2 (штамм альфа), при одномоментном внесении соединений и вируса.
Результаты. Синтезированы производные аминокислоты гистидина с карбоциклами и кластерными анионами бора, и исследована их противовирусная активность в отношении коронавируса SARS-CoV-2 *in vitro*. На клеточных культурах показано, что производные гистидина с карбоциклами и кластерным анионом бора $[B_{10}H_{10}]^{2-}$ обладают способностью подавлять репликацию вируса. Также была показана возможность увеличения растворимости субстанции в водных средах за счёт образования хлоргидрата или натриевой соли.
Обсуждение. Соединение I 2HCl*H-His-Rim проявляло некоторый эффект подавления репликации вируса SARS-CoV-2 при вирусной нагрузке 100 доз и концентрации 31,2 мкг/мл. Наиболее очевидным объяснением противовирусного действия соединения I на угнетение репликации SARS-CoV-2 в эксперименте *in vitro* могут являться слабоосновные свойства, которые проявляет это соединение.
Заключение. Представленные синтетические соединения проявили умеренную противовирусную активность в отношении варианта коронавируса SARS-CoV-2. Полученные соединения могут быть использованы в качестве модельных структур для создания нового препарата прямого действия против современных штаммов коронавирусов.

Ключевые слова: *производные адамантана; аминокислота гистидин; противовирусная активность; SARS-CoV-2; кластерные анионы бора*

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Участие авторов: Гараев Т.М. – идея использования производных кластеров аниона бора с аминокислотными остатками в качестве противовирусных средств, молекулярный дизайн структуры будущего ингибитора, анализ и предсказание свойств молекул карбоциклов и борного кластера, замещенных аминокислотными остатками, методами молекулярного моделирования *in silico*, синтез соединений остатка гистидина с карбоциклическими фрагментами, введение гистидинового фрагмента в борный кластер за счёт проведения реакции с замещенным производным $[2\text{-}B_{10}H_9OC_4H_8COOH]^{2-}$, написание текста статьи; Гребенникова Т.В. – общее руководство и разработка концепции исследования нового класса соединений, постановка задачи и контроль над выполнением эксперимента, согласование условий постановки эксперимента и анализ результатов; Авдеева В.В. – синтез исходных солей декагидро-*клозо*-декаборатного аниона, проведение многостадийной функционализации борного кластера: введение в кластер оксониевого заместителя с образованием $[B_{10}H_9OC_4H_8]^-$, его нуклеофильное раскрытие, гидролиз полученного замещенного производного $[B_{10}H_9OC_4H_8CN]^{2-}$ с образованием *клозо*-декаборатного аниона с COOH группой $[B_{10}H_9OC_4H_8COOH]^{2-}$, редактирование текста статьи, перевод на английский язык; Лебедева В.В. – контроль за выполнением эксперимента, согласование условий постановки и результатов эксперимента по синтезу целевого соединения, определение стратегии проведения эксперимента *in vitro*, анализ результатов эксперимента, редактирование текста статьи; Ларичев В.Ф. – разработка концепции и проведение эксперимента *in vitro*: изучение

специфической активности производных в отношении пандемического штамма коронавируса SARS-CoV-2 на клетках культуры ткани Vero E6.

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Introduction

Coronaviruses are zooanthroponotic viruses capable of infecting humans and animals. Modern strains of coronaviruses (CoVs) are capable of causing severe and fatal illness in humans. The emergence of Severe Acute Respiratory Syndrome (SARS) in 2002 and Middle East Respiratory Syndrome (MERS) in 2012 highlighted the ability of CoVs to cause fatal diseases in humans [1–4].

During the current COVID-19 pandemic [5, 6] caused by the novel coronavirus SARS-CoV-2, containment measures and vaccination have slowed the spread of the infection, but have not been able to completely prevent the disease among healthcare workers, patients and the entire population of the planet.

In connection with the outbreak of new infectious diseases caused by various pathogenic viruses and the development of resistance to classical antiviral drugs, pharmaceutical companies and numerous research groups are looking for new antiviral agents with unique chemical and physical properties [7, 8]. To date, there is no approved therapeutic agent against any human coronavirus, but a number of antiviral compounds under investigation are currently undergoing clinical trials [9-14]. Creating new antiviral drugs against COVID-19 is challenging and requires significant time and effort to develop and validate.

Currently, a number of low molecular weight compounds are being developed as potential inhibitors of CoVs replication aimed at various stages of the replication cycle [15]. Some compounds, in particular inhibitors of the main CoVs protease, are currently undergoing the final stages of clinical trials and are awaiting approval from the pharmaceutical committee. This class of compounds are peptidomimetics capable of inhibiting the enzyme 3-chymotrypsin-like protease (3CLpro). 3CLpro cleaves the precursor peptide at 11 sites into individual viral proteins of the new CoVs virion [16]. Another class of antiviral compounds is represented by nucleoside analogues that mimic naturally occurring nucleosides to suppress viral RNA-dependent DNA polymerase [17].

An alternative protein target can be small viral proteins capable of forming pores in cell membranes and virus envelopes, viroporins. Viroporin inhibitors can form a new class of antiviral drugs that can act as an independent drug, as in the case of Remantadine against influenza A virus, and possibly as part of complex therapy. SARS-CoV-2 E proteins have a polypeptide length of 76 amino acids and have one α-helical transmembrane domain [18, 19]. Proteins E are capable of forming pentameric structures with the function of ionic conduction [20]. Protein E from CoV appears to be the most likely target for inhibitor molecules based on adamantyl amino acids. The adamantane carbocyclic cage as a lipophilic agent in this case plays the role of a carrier for an amino acid residue or another physiologically important compound. The adamantane cage is a highly hydrophilic residue, therefore, in some cases, it can be replaced by other carbocycles, such as norbornene or cyclohexane [21].

We have previously shown that dichloride L-histidyl-aminoethyladamantane (2HCl*H-His-Rim) exhibits an antiviral effect against influenza A virus strains resistant to Amantadine and Remantadine. Moreover, this compound was an inhibitor of the function of the M2 ion channel of the influenza A virus [22]. Viroporin M2 is vital for the influenza A virus to infect the cell. This is an ion channel built into the viral envelope that selectively conducts hydrogen ions through itself from the cell into the virus. The virus enters the host cell enclosed in endosomes (membrane structures) as a kind of bubbles. At a certain value of the acidity of the environment, the M2 protein is activated and begins to pump hydrogen ions, lowering the pH inside the viral particle and thereby causing its disintegration to release the genetic material of the virus into the cytoplasm of the host cell.

The influenza A virus M2 protein and the CoV E protein have a similar secondary structure, a comparable amino acid sequence length, and also play an important role in the virus assembly step. This suggests that these two viroporins should be sensitive to homologous synthetic compounds. Thus, there are all prerequisites for achieving the effect of suppressing the function of the channel formed by the E protein of the coronavirus, carbocyclic derivatives of the histidine amino acid residue.

The aim of the study is to identify the antiviral properties of histidine derivatives with cage substituents in relation to the pandemic strain of SARS-CoV-2 coronavirus *in vitro.*

Materials and methods

Synthetic compounds

The compounds under consideration were obtained by the methods of organic and peptide synthesis described by us earlier [21, 23, 24]. **Fig. 1** shows the structures of compounds that exhibited antiviral activity against modern strains of influenza A virus. L-histidyl-aminoethyladamanantane dihydrochloride (compound **I**, 2HCl*H-His-Rem) [25]; a derivative of the *closo*-decaborate anion with a histidine methyl ester residue as a pendant substituent, separated from the boron cluster by a spacer -O$(CH_2)_4$CO- (compound **II**, $Na_2[B_{10}H_9$-O$(CH_2)_4$CO-His-OMe]) [26, 27]; histidyl-dicyclohexyl

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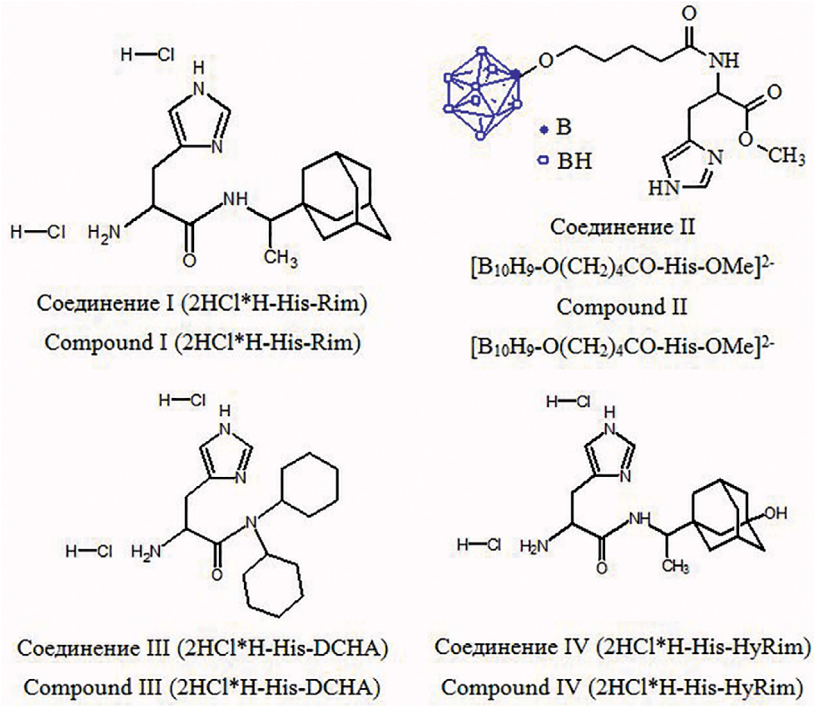

Fig. 1. Synthetic compounds of histidine residue with bulky cage substituents.
Рис. 1. Синтетические соединения остатка гистидина с объёмными каркасными заместителями.

amide dihydrochloride (compound **III**, 2HCl*H-His-DCHA); histidyl-3-hydroxy-aminoethyladamantane dihydrochloride (compound **IV**, 2HCl*H-His-HyRim). Compounds **III** and **IV** are new, but were obtained in a manner similar to that of compound I.

Virus

The studies used a strain of human coronavirus SARS-CoV-2, passage 4, with an infectious activity of 106 TCID50/ml for Vero E6 cells deposited and the State Collection of Viruses of the Russian Federation of the Federal State Budgetary Institution "National Research Center for Epidemiology and Microbiology named after N.F. Gamaleya" of the Ministry of Health of Russian Federation under the number No. 1301/2 GKV.

GISAID: 421275 EPI ISL

Russia/Moscow PMVL-1/2020 GISAID Clade GR

Clade 20B

The SARS-CoV-2 virus was passaged and titrated on a Vero E6 monolayer culture. Infectious titer was determined by the standard titration method and calculated according to the method of Reed and Mench. The culture fluid lysate was poured into aliquots and kept at –70 ± 10 °C until the study.

Cell culture

In the experimental work, we used a continuous cell line of African green monkey (*Chlorocebus aethiops*) kidney cells Vero E6 of the All-Russian Collection of Cell Cultures at the Federal State Budgetary Institution "National Research Center for Epidemiology and Microbiology named after N.F. Gamaleya" of the Ministry of Health of Russian Federation.

Cell cultivation was carried out on DMEM medium. Vero E6 cells were placed at 12,000 cells/well in 96-well culture flat-bottomed plates in a volume of 100 µl of freshly prepared DMEM medium. Cultivated for 24 hours at a temperature of 37 °C in an atmosphere of 5% CO_2.

Evaluation of the cytotoxic effect of compounds

An experiment to evaluate the cytotoxic effect on cell culture was carried out in the range of 12 concentrations of the drug from 2500.0 to 0.5 µg/ml in steps of two by triturating the initial concentration in the wells of a 96-well plate. After cells were incubated with drugs for 96 h at 37 °C in an atmosphere of 5% CO_2, the state of the cell monolayer was visually assessed by comparing it with cell control (without compounds) according to a four-cross scheme, where four crosses correspond to 100% cell death, two crosses correspond to 50% cytotoxic concentration (CC_{50}). Thus, based on the comparison of the state of the cell monolayer without preparations with cells containing the compounds at the appropriate concentration, the cytotoxic effect of the compounds on the cell monolayer was determined.

Evaluation of the antiviral activity of compounds

The assessment of viral production by cytopathic effect (CPE) was carried out on the basis of cell viability analysis using microscopy in order to visually determine the boundaries of viral cell damage, as well as to control the toxicity of substance doses.

An experiment to assess cell viability in the test for antiviral efficacy was carried out in the concentration range of the drug 2500.0 to 0.5 µg/ml by titrating the initial concentration in the wells of a 96-well plate. The

antiviral activity of the compounds was assessed visually under a microscope 96 h after infection by inhibition of the CPE of the virus in a Vero E6 cell culture.

Results

Compound **I** showed a stable effect of suppressing the replication of the SARS-CoV-2 virus at a very narrow range of non-toxic concentrations of 75.0–30.0–μg/ml and a viral load of 100 doses. The 50% cytotoxic dose was about 70 μg/ml, the concentration to achieve a stable antiviral effect was about 31 μg/ml (**Table**). To reduce toxicity and improve the antiviral properties of compound **I**, the adamantane carbocycle was replaced by two cyclohexane residues in the form of dicyclohexylamine (2HCl*H-His-DCHA, compound **III**). The toxicity of compound **I**, indeed, was reduced several times, CC_{50} was 312 μg/ml. However, the antiviral activity of compound **III** is significantly lower than for compound **I** and is about 156 μg/ml. At the same time, the antiviral effect of the compound was observed only at the border between toxic and non-toxic concentrations. When the concentration was lowered below 156 μg/ml, the antiviral effect of compound **III** disappeared. Moreover, it should be noted that the solubility of compound **III** deteriorated compared to completely water-soluble compound **I**. To dissolve compound **III**, a composition of 10% acetonitrile, 30% of 4% PEG 1150 solution in water, and 60% DMEM medium was used. Another way to increase the affinity of a substance for the target protein to form additional hydrogen bonds with the amino acid residues of the inner pore of the protein E channel was to add a hydroxyl group to the para position in the adamantane carbocycle (compound **IV**).

The antiviral properties of compound **IV**, like compound **III**, were manifested at higher concentrations compared to compound **I**. Moreover, compound **IV** was much less soluble in the aqueous medium than compound **I**. This modification did not lead to an improvement in the antiviral properties of compound **I**. At high active concentration of the compound, there are no prospects for obtaining a high chemotherapeutic index (at least in in vitro experiments), which is an important factor for a candidate compound. From previous studies comparing the histidine compound with a boron cluster anion (compound **II**) and compound **I** in relation to influenza A virus, it was shown that compound **II** was slightly superior in antiviral properties to compound **I** [23], the 50% inhibitory dose was 3.2 μg/ml versus 7.5 μg/ml for compound **I**. It is important that compound **II** has a high aqueous solubility, like compound **I**, since it is a disodium salt. Tests of the antiviral properties of compound **II** against SARS-CoV-2 showed their presence in the concentration range of 19.5–4.9 μg/ml.

Table. Biological properties of histidine derivatives with cage substituents

Таблица. Биологические свойства соединений гистидина с каркасными заместителями

№	Compound Соединение	CC_{50}, mkg/ml CC_{50}, мкг/мл	IC_{50}, mkg/ml IC_{50}, мкг/мл	SI
1	Compound I Соединение I 2HCl*H-His-Rem	78,1	31,2	2,5
2	Compound II Соединение II $Na_2[B_{10}H_9$-$O(CH_2)_4CO$-His-OMe]	19,5	9,8	2
3	Compound III Соединение III 2HCl*H-His-DCHA,	312,5	156,3	2
4	Compound IV Соединение IV 2HCl*H-His-HyRim	312,5	156,3	2

Discussion

Compound **I** 2HCl*H-His-Rem showed an antiviral effect against influenza A virus strains resistant to Amantadine and Remantadine. This compound was an inhibitor of the function of the M2 ion channel of the influenza A virus [22]. There were some prerequisites that it would be possible to achieve the effect of suppressing the function of the channel formed by the E protein of the coronavirus. Protein E is poorly represented on the surface of the virion, but it is present in large quantities in infected cells. Protein E is mainly distributed in intracellular membranes between the endoplasmic reticulum (ER) and the cisterns of the Golgi complex. In these places, it is involved in the assembly of the virus and intracellular transport. Compound **I** showed some effect of suppressing SARS-CoV-2 virus replication at a viral load of 100 doses and a concentration of 31.2 μg/ml. J.S. Kim et al. [28] in their in silico studies with the well-known ion channel blocker amantadine (1-aminoadamantane) hypothesized that amantadine blocks the SARS-CoV-2 ion channel, preventing the release of the viral nucleus into the cell cytoplasm. The BIO-HPC Achilles Blind Docking Server online service was used to perform molecular computer simulation of the docking of compound **I** and the crystallographic structure of the protein channel E. This tool performs an exhaustive series of docking calculations over the entire surface of a protein to find sites with the best affinity for the target protein. After calculating the similarity, this service groups the results according to the spatial overlap of the received positions. The structural model of the ligand (drug) was generated in HyperChem 8.0.8 software (Hypercube). Molecular modeling of the docking of the protein and ligand surfaces on the model of the transmembrane region of viroporin E (PBD: 5X29) resulted in a positive solution, namely, the detection of compound **I** at the channel constriction site surrounded by Phe26 and Ala22 residues (**Fig. 2**). Compound **I** sterically blocks the channel pore, which is composed of five subunits of protein E, forming a non-covalent interaction with the conjugation ring of phenyl radicals from Phe26 residues. The inhibitor compound is also found between the helices of individual strands. Figure 2 shows five positions of the molecules of compound **I** in the intercoil space, and these solutions are not equivalent for each strand.

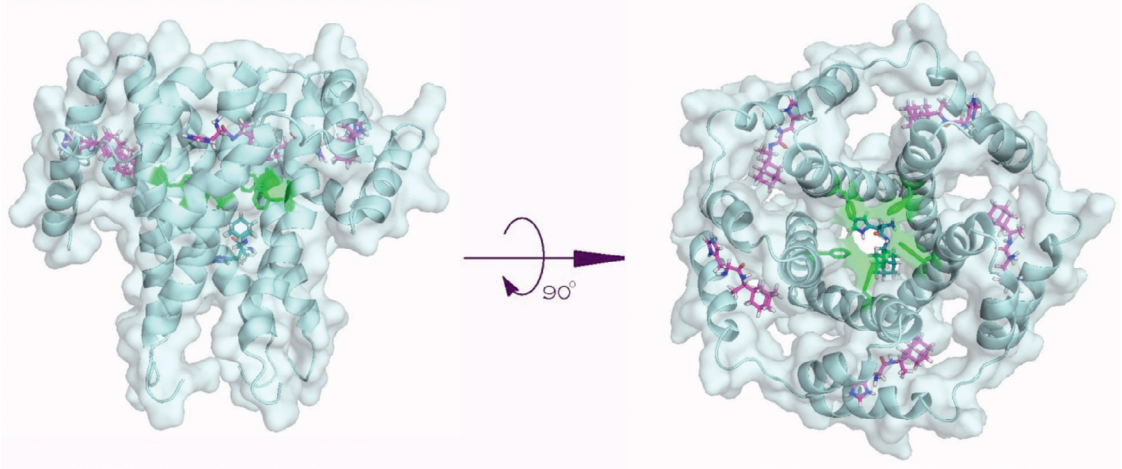

Fig. 2. Complex of compound I with the transmembrane domain of the E protein pentamer (viroporin) of the SARS-CoV-2 coronavirus (PDB: 5×29). Top and side views are shown. The figure shows the positions of the ligand in the channel pore and in the intercoil space of individual subunits. The conjugation of phenyl rings from the Phe26 residues of each chain is shown, which closes and opens the channel for directed transport of monovalent ions.

Рис. 2. Комплекс соединения I с трансмембранным доменом пентамера белка E (виропорином) коронавируса SARS-CoV-2 (PDB: 5×29). Представлены виды сверху и сбоку, показаны положения лиганда в поре канала и межспиральном пространстве отдельных субъединиц, сопряжение фенильных колец от остатков Phe26 каждой цепи, которое закрывает и открывает канал для направленного транспорта одновалентных ионов.

Another explanation for the antiviral effect of compound **I** on the inhibition of SARS-CoV-2 replication in an in vitro experiment may be the weakly basic properties that compound **I** exhibits. It is known that an increase in the pH of the endosomal content prevents the unpacking of viral particles. The open amino group and the imidazole group act as a weak base, while the carbocycle is able to migrate across membranes as a hydrophobic agent. In this case, the mechanism of action of compound **I** is rather similar to the action of the drug hydroxychloroquine sulfate, which was used to treat patients in the first months of the COVID-19 pandemic [29]. It is important to note that as a result of molecular modeling for compound **III** on the same viroporin E model, a positive solution was obtained, namely, the detection of the molecule at the constriction site of the pumping mechanism (Phe26). However, the experiments carried out in vitro for compounds **I** and **III** do not confirm the equivalent effect of these compounds on virus replication. Most likely, the antiviral effect is achieved only due to the weakly basic properties of the histidine residue and, therefore, is manifested only in high concentrations of compounds in the well of the plate.

The cytotoxic effect of compound **II** was higher than for compound **I**; on the other hand, the effective concentration was lower. Complete suppression of CPE of the virus by compound **II** was observed at a concentration of 9.8 μg/ml versus 31.2 μg/ml for compound **I**. The mechanism of action of compound **II** has not been established. It can be assumed that the detected antiviral properties of compound **II** are probably associated with the steric features of the molecule, which make it possible to block the function of viroporin. SARS-CoV viroporin E is known to have ion channel activity for monovalent cations with a 10-fold preference for sodium ions compared to potassium ions [30]. Being inside the pore of the channel, the anion of compound **II** has the ability to capture positively charged ions into the coordination sphere, which disrupts the function of viroporin aimed at creating favorable homeostasis, maturation, and intracellular transport of viral proteins.

Conclusions

The presented synthetic compounds showed moderate antiviral activity against the SARS-CoV-2 variant of the coronavirus. The resulting compounds can be used as model structures for creating a new direct-acting drug against modern coronavirus strains. Protein E of the coronavirus appears to be the most likely target for inhibitor molecules based on adamantyl amino acids. Potentially, these results can be applied to other viroporins of RNA viruses, such as Vpu from human immunodeficiency virus type 1, p7 from hepatitis C virus, E5 from human papillomavirus and bovine diarrhea virus (BVDV), NSP-4 from rotavirus and other viruses. ion channels.

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