ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2017, Vol. 43, No. 5, pp. 517–525. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © V.A. Shibnev, P.G. Deryabin, T.M. Garaev, M.P. Finogenova, A.G. Botikov, D.V. Mishin, 2017, published in Bioorganicheskaya Khimiya, 2017, Vol. 43, No. 5, pp. 491–500.

Peptide Carbocyclic Derivatives as Inhibitors of the Viroporin Function of RNA-Containing Viruses

V. A. Shibnev, P. G. Deryabin, T. M. Garaev[1], M. P. Finogenova, A. G. Botikov, and D. V. Mishin

Ivanovskii Institute of Virology, Gamaleya Federal Research Center for Epidemiology and Microbiology, Ministry of Health of Russia, Moscow, 123098 Russia

Received September 2, 2016; in final form, April 10, 2017

Abstract—New carbocyclic derivatives of amino acids, peptides, and other compounds have been synthesized, and their antiviral activity toward the influenza A/H5N1 and hepatitis C viruses has been studied in vitro. It has been shown that the aminoacyl derivatives of aminoadamantane are capable of inhibiting the replication of the highly virulent strain of the avian influenza virus (H5N1), which is resistant to aminoadamantanes amantadine and rimantadine. The effect of the configuration of the carbocyclic moiety of the dipeptide H-Pro-Trp-OH on the antiviral properties toward the hepatitis C virus has been studied. The cyclohexyloxycarbonyl derivative of the H-Pro-Trp-OH dipeptide strongly inhibited the replication of HCV in vitro. Some compounds have been found to exhibit a high virucidal activity toward influenza A/H5N1 and HCV virions.

Keywords: A/H5N1 influenza virus, adamantane derivatives, peptides, hepatitis C virus, antiviral activity

DOI: 10.1134/S1068162017050132

INTRODUCTION

Viral ion-selective channels proteins are involved in many stages of the virus replication cycle. These channels are localized in the virus envelope or the membranes of the host cell and play an important role in the penetration of viruses into the cell, as well as the assembly and exit of newly formed virions. Viral ion channels are not always required for virus replication, although their involvement usually significantly contributes to an increase in the number of virions. The family of these proteins received the name viroporins; it involves proteins containing at least one hydrophobic transmembrane (TM) domain in the form of the α-helix [1]. As a rule, viroporins are small proteins (50–120 amino acid residues long), which tend to form homooligomers. The studies of the molecular architecture of viral ion channels showed that most of them are formed by tetramers although penta- and hexameric structures are also characteristic of viroporins. A representative of tetrameric ion-selective viroporins is the channel M2 of the influenza A virus. This unique viroporin is specific to the transport of hydrogen ions into the virion, whereas the majority of viroporins exhibit moderate ion selectivity, i.e., do not display preference for a particular ion. The pentameric viroporin, the protein Vpu from HIV-1, shows moderate cation selectivity in NaCl and KCl electrolyte solutions [2]. This holds true also for the HCV hexameric viroporin p7, which transports monovalent cations (Na^+ and K^+) [3].

The high ion selectivity of the influenza A virus M2 viroporin is due to a special structure of its TM domain, which contains histidine residues at position 37 (His37) in the form of the tetrameric conjugation of their imidazole rings. Protons from the intracellular environment protonate three or all four imidazole rings of histidines. This leads to a conformational change due to electrostatic repulsion, which widens the channel in the region of His37 and enables the protons to penetrate the virus envelope [4]. The indole group of tryptophan 41 plays an important role in the stabilization of the open state of the channel pore. Thus, the molecular model of the functioning of the M2 ion channel represents a "sluice" mechanism [5].

Among the first inhibitors of the influenza A virus M2 proton channel to be investigated was amantadine (Symmetrel, 1-adamantylamine hydrochloride). It has been used in clinical practice against influenza A virus since 1966 [6]. Later, in 1985 it was shown that some mutations in the hydrophobic sequence of the M2 protein can cause the resistance of the virus to amantadine and its analogue Rim (Flumantadine, 1-(1-adamantyl)ethylamine hydrochloride). These findings suggested that the M2 protein may be a target

[1] Corresponding author: phone: +7 (499) 190-30-43; e-mail: tmgaraev@gmail.com.

Abbreviations: Amt, 1-aminoadamantane; Ad, 1-adamantanecarboxylic acid; Cho, (cyclohexyloxy)carboxylic acid; IBCF, isobutylchlorformiate; Met(O_2), methionine sulfone; Nor, 2-norbornene carboxylic acid; Nrb, 2-norbornan carboxylic acid; NMM, *N*-methylmorpholine; Rim, rimantadine (1-(1-adamantyl)ehylamine hydrochloride); TEA, 3-(2-thienyl)propenoic acid; Tha, 2-tetrahydrofuran carboxylic acid; Qln, quinoline-2-carboxylic acid; HCV, hepatitis C virus.

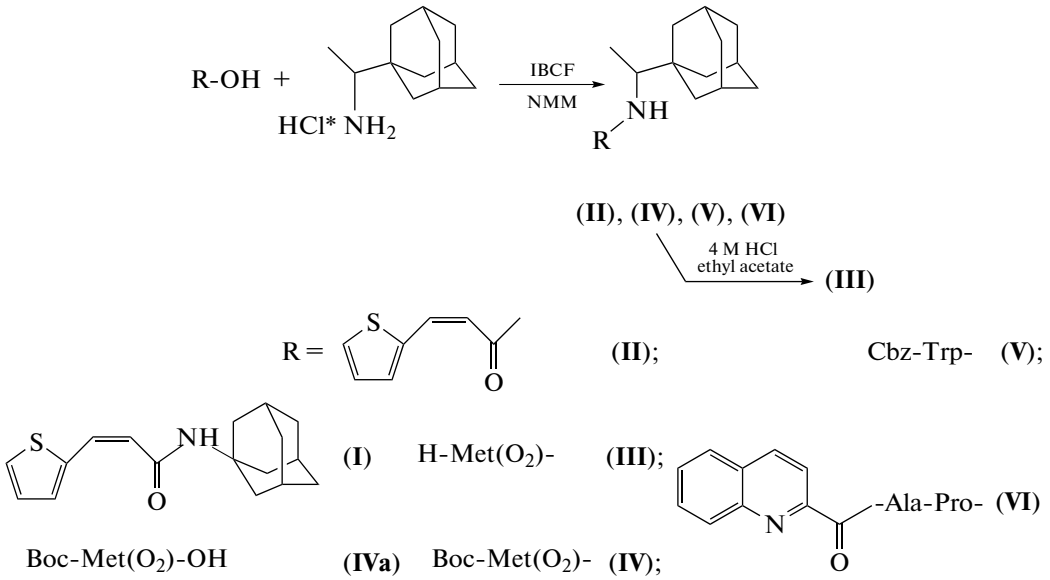
for the preparations of the adamantane series [7]; in 1992, this suggestion was confirmed [8].

Recent investigations have shown that the ion activity of the hexameric channel protein p7 of HCV can also be inhibited by adamantane preparations in vitro [9, 10]. This gives promise that the p7 channel can be an adequate target for adamantane-based anti-HCV drugs. The nonstructural HCV p7 protein consists of 63 amino acid residues and has two TM domains (TM1 and TM2) [11]. This viroporin is predominantly localized in intracellular membranes; in experiments in vitro, it displays the function of ion channels necessary for the virus assembly and optimal exit from infected cells by changing the acid–alkaline equilibrium in intracellular vesicles. The membrane protein p7 is a new target for the antiviral therapy of HVC [12].

RESULTS AND DISCUSSION

The resistance of the virus to amantadine and Rim results from a mutation in the amino acid sequence of proteins in the M2 proton channel of the influenza A virus. It should be noted that Rim and amantadine preparations exhibit cross-resistance. One of the ways of restoring the antiviral properties of adamantane preparations is the introduction of additional functionally active groups into their structure. In the present study, substituted amino acids, peptides, and TEA were proposed as the sources of these groups. The residues of amino acids and peptides can be attached to Rim or amantadine molecules, which have one amino group, by the method of mixed anhydrides [13, 14].

As a result, we synthesized several derivatives of adamantane and some other carbocycles, such as norbornene, cyclohexane, and Qln (Scheme 1):



Scheme 1. A general scheme of the synthesis of rimantadine derivatives. Compound (**I**) was synthesized in a similar way but with the use of 1-aminoadamantane as an amino component instead of rimantadine.

N-[3-(2-thienyl)propenoyl]-(1-adamantyl)amide (TEA-Amt (**I**));

N-[3-(2-thienyl)propenoyl]-1-adamantaylethylamide (TEA-Rim (**II**)); methionyl(dioxide)-(1-adamantaylethyl)amide hydrochloride (HCl*Met(O_2)-Rim (**III**));

N-Boc-methionyl(dioxide)-(1-adamantaylethyl)amide (Boc-Met(O_2)-Rim (**IV**));

N-Z-L-tryptophyl-(1-adamantylethyl)amide (Z-Trp-Rim (**V**));

quinaldine-2-oyl-L-alanyl-L-prolyl-(1-adamantylethyl)amide (Qln-Ala-Pro-Rim (**VI**)).

The antiviral activity of these compounds was assessed in vitro using a highly pathogenic strain of the avian influenza virus A/H5N1, which is resistant to Rim, on models of the African green monkey kidney (Vero-E6) and the porcine embryo kidney (SPEV) cell lines.

Antiviral activity of compounds toward the A/duck/Novosibirsk/56/05 (H5N1) virus. The antiviral activity of a compound as a rule involves both its virucidal and antiviral properties, i.e., the capacity to inhibit a particular stage of virus replication, from the adsorption of a virus on the cell and its penetration into the cell to the influence on the assembly and exit of the virus from the infected cell. Therefore, the antiviral activity of a compound being examined is tested by treating a cell monolayer with this compound prior to the infection with the virus (the prophylactic effect of the compound), immediately at the time of infection (the treatment-and-prophylactic effect), and some time after the infection (the treatment effect). The antiviral effect of the compound is estimated from the fraction of viable infected cells. In the experiment, the viability (percent of surviving cells) of Vero-E6 (or SPEV) cells infected with a highly virulent strain of the

Table 1. Antiviral activity of compounds toward the infection caused by the highly pathogenic influenza virus A/H5N1

Time of administration of compound	Indicator of the effect of compound	Effective concentration (μg/mL) for compounds:						
		(I)	**(II)**	**(III)***	**(IV)***	**(V)***	**(VI)**	rim hydrochloride
6 h prior to infection	CD_{50}	>6.25	>50.0	25.0	100.0	100.0	>12.5	>12.5
	ID_{50}	0.2	1.56	<3.12	0.2	0.2	<1.56	>12.5
	ID_{95}	0.4	3.12	3.12	0.4	0.4	1.56	>12.5
At the instant of infection of cells	CD_{50}	>6.25	>50.0	>50.0	>25.0	>100.0	>25.0	25.0
	ID_{50}	0.4	<6.25	<3.12	0.4	0.4	1.56	>25.0
	ID_{95}	0.8	6.25	3.12	0.8	0.8	3.12	>25.0
6 h after the infection of cells	CD_{50}	>12.5	>50.0	50.0	100.0	>100.0	>12.5	25.0
	ID_{50}	6.25	<6.25	12.5	<6.25	0.8	1.56	25.0
	ID_{95}	12.5	1.56	25.0	12.5	1.56	3.12	50.0

* Experiment was performed using the SPEV cell culture.
ID_{50}, the inhibitory dose 50: a minimal dilution of a preparation that provides the protection of 50% of monolayer cells;
CD_{50}, the cytotoxic dose 50: a dilution of a preparation that causes the death of 50% of monolayer cells;
ID_{95}, the inhibitory dose 50: a minimal concentration of a compound that provides the protection of 95% of cells.

influenza virus A/duck/Novosibirsk/56/05 (H5N1) was studied using different schemes of the administration of the compounds synthesized.

It is seen from the data in Table 1 that the Rim derivative with thienylpropenoic acid (**II**) effectively protected a monolayer of Vero-E6 cells with all schemes of the administration of the compound: $ID_{50} < 0.8$ μg/mL prior to, and at the time of infection and $ID_{50} = 6.25$ μg/mL with the treatment scheme of administration. The Rim derivative with TEA (**I**) showed a similar efficacy with all schemes of the administration; $ID_{50} = 1.56$ μg/mL with the prophylactic scheme and less than 6.25 μg/mL with the treatment-and-prophylactic and treatment schemes.

The antiviral activity of synthetic compounds (**III**), (**IV**), and (**V**) was estimated from their capacity to protect a monolayer of SPEV cells from the cytopathic action of the virus. The compounds were highly effective when being administered according to the treatment-and-prophylactic and treatment schemes. The ID_{50} value was less than 0.8 μg/mL for compounds (**III**) and (**V**) and less than 3.12 μg/mL for (**IV**). If these compounds were administered according to the treatment scheme, the inhibitory activity decreased.

The *N*-quinaldinoyl derivative of Rim, dipeptide amide (**VI**), effectively protected Vero-E6 cells with all schemes of administration: the ID_{50} value was 1.56 μg/mL with the treatment-and-prophylactic scheme and somewhat less with the prophylactic and treatment schemes.

Virucidal activity of compounds toward the A/duck/Novosibirsk/56/05 (H5N1) virus. The virucidal activity of a compound is associated with the direct inactivating effect on virions incorporated into the viral population with the result that the infectivity of a virus is partially or completely lost. To test the virucidal properties of a compound, it is sufficient to incubate a mixture of a virus and the compound for a particular time and to test then the infective capacity of the virus without the compound by the titration in cell cultures. A titer of a virus for cell cultures is a minimal dilution of the virus that causes the death of 50% of cells of the monolayer, expressed as the common logarithm ($logTCID_{50}$). A reliable decrease in the infectivity of a virus in the scale of common logarithms by 1.0 and more or its complete loss as compared with the virus without a substance indicates the virucidal activity of the compound being tested. The virucidal activity of the synthetic compounds is demonstrated in Table 2.

It is easily seen from Table 2 that compounds (**I**) and (**II**) possess a high virucidal activity. When the virus-containing material came in contact with the compounds, the virus completely lost its infectivity within 20 min. Compound (**V**) also exhibited a high virucidal effect: the decrease in the infectious titer was by four and more logarithmic units (10000 times) relative to the viral control ($logTCID_{50/0.2} = 7.0$ per 200.0 μL of the virus-containing material). Compound (**IV**) had a moderate virucidal activity: the decrease in the infectious titer was approximately by four units (10000 times) relative to the control ($logTCID_{50/0.2} = 7.7$). The virucidal effect of compound (**III**) was much weaker. We failed to reveal the virucidal effect of compound (**VI**). The decrease in the infectious titer was less than one logarithmic unit.

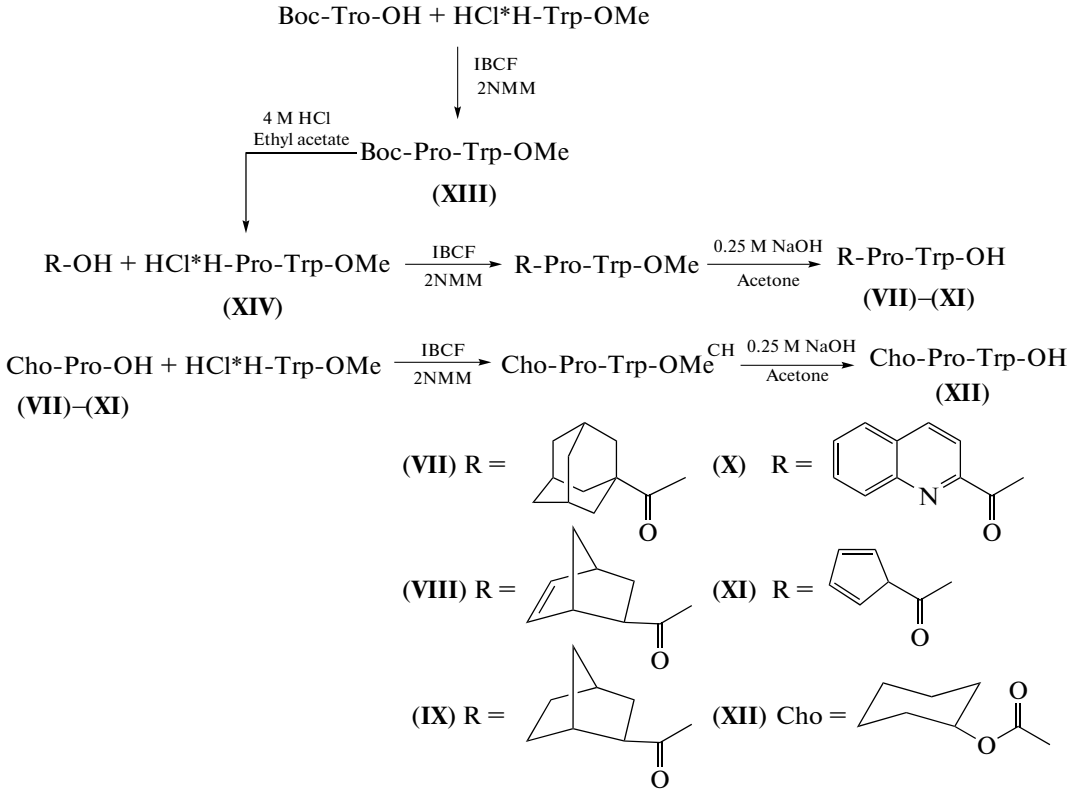
The antiviral activity of the derivatives of dipeptide H-Pro-TRP-OH toward the infection caused by HCV with different schemes of administration. We have shown earlier that the adamantane carbocyclic peptide derivatives of Rim are capable of inhibiting the replication of HCV in vitro [15]. These compounds represented Boc-lysine coupled to the adamantane carbocycle through a spacer of glycine residues (from one to three in a chain). The compound with three glycine

residues in the spacer Boc-Lys(Boc)-Gly_3-Rim showed the highest activity.

The studies performed in silico showed that, in the majority of models, Boc-Lys(Boc)-Gly_3-Rim is bound to several chains of the p7 ion channel. The interaction of this ligand with the p7 target protein was simulated using the program Patchdock v.1.3. Molecular Docking Algorithm (BioInfo3D), which makes it possible to perform the docking of molecules in silico. The model of the p7 channel was obtained from the open database of 3D protein structures (Protein Data Bank, code 2M6X), and the structure of the ligand was generated by molecular simulation methods using the program HyperChem v.8.0.0 (Hypercube, Inc.) (Figs. 1c, 1d). This model was built by the PM3 semiempirical method of quantum mechanical calculations.

The results of the solution presented in the figure show that there are four points of binding to the amino acid residues of the viral channel proteins. In all, the protein has six chains (a hexameric model was used), and four of these chains are involved in the docking, namely: His57 residues from different chains interact; His17 interacts with the α-amino group of lysine, and Gly22 interacts with the methyl group of Rim. Histidine residues are very important for the operation of the ion channel; they are responsible for the selectivity of the ion pump and unidirectivity of the ion flow [4]. The compound Boc-Lys(Boc)-Gly_3-Rim has a bent configuration, and this stereometry and the length of the spacer composed of glycine residues probably contribute to the formation of a stable complex of the inhibitor molecule with the target protein.

In the present work, we made an attempt to study how the carbocyclic backbone of the peptide derivative affects its capacity to inhibit the replication of HCV in vitro. The following carbocyclic carboxylic acids were used: 1-adamantanecarboxylic (Ad-OH), 2-norbornene carboxylic (Nor-OH), 2-norbornan carboxylic (Nrb-OH), quinoline-2-carboxylic (Qln-OH), 2-tetrahydrofuran carboxylic (Tha-OH), and cyclohexyloxycarboxylic (Cho-OH) acids. With the use of these acids, the dipeptide H-Pro-Trp-OH was acylated by the methods of classical peptide synthesis in solution (Scheme 2). The construction of the dipeptide was chosen not accidentally: the unique electron properties of the indole group of tryptophan in combination with the conformational features of the proline residue should have to create a multifunctional group with the hydrophobic properties of the carbocyle.



Scheme 2. A general scheme of the synthesis of carbocyclic derivatives of dipeptide H-Pro-Trp-OH. Compound (**XII**) was synthesized based on Cho-Pro-OH (see the Experimental section).

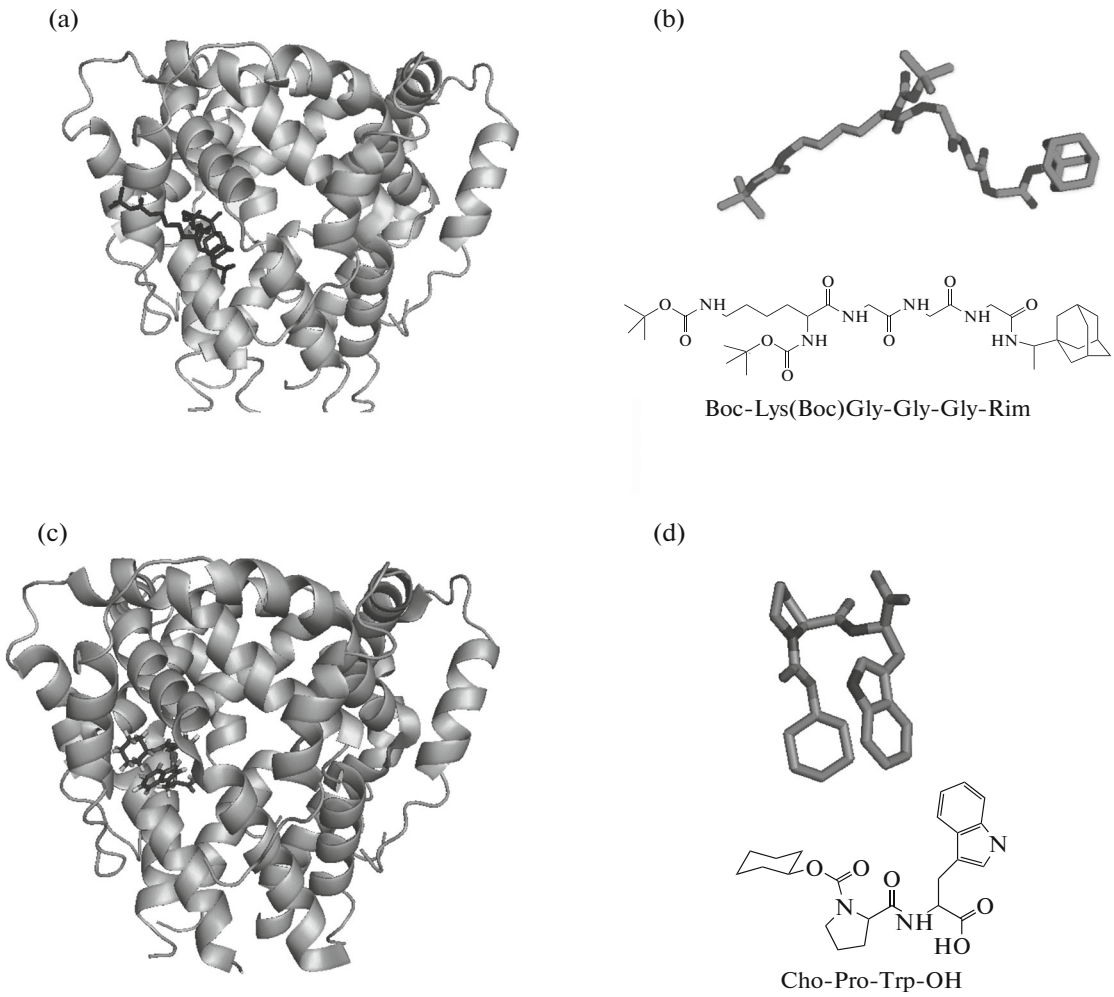
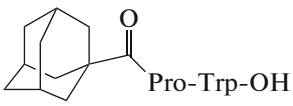
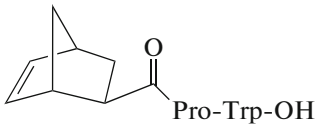


Fig. 1. Molecular models of the inhibitors Boc-Lys(Boc)-Gly_3-Rim [15] (c) and Cho-Pro-Trp-OH (d) and their complexes with the hexameric protein channel p7 of HCV ((a) and (b), respectively).

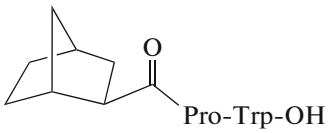
Thus, the following compounds were synthesized:



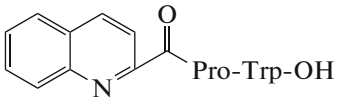
Ad-Pro-Trp-OH (**VII**)



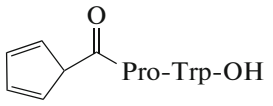
Nor-Pro-Trp-OH (**VIII**)



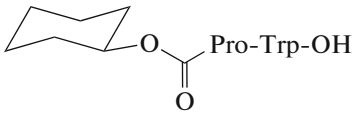
Nrb-Pro-Trp-OH (**IX**)



Qln-Pro-Trp-OH (**X**)



Tha-Pro-Trp-OH (**XI**)



Cho-Pro-Trp-OH (**XII**)

It is seen from Table 3 that all compounds were ineffective or little effective with the treatment scheme of the administration. In the prophylactic and treatment-and-prophylactic schemes of the administration of the virus and compounds, only one combination of the carbocycle with the dipeptide Cho-Pro-Trp-OH (**XII**) merits notice. The ID_{50} values for this compound were 0.3, 0.6, and 12.0 μg/200 μL according to the three schemes of administration. Among the other compounds, the derivative Ad-Pro-Trp-OH (**VII**) is

Table 2. Virucidal activity of compounds (**I**)–(**VI**) toward the highly pathogenic influenza virus A/H5N1

Indicator	(I)	(II)	(III)	(IV)	(V)	(VI)	Rim
$logTCID_{50}$/0.2 mL	0	0	7.0	4.8	2.9	8.0	8.5
log of decrease in infectivity	7.7	6.6	1.5	3.7	4.1	0.5	0
Without preparation (control)	7.7	6.6	8.5	8.5	7.0	8.5	8.5

$logTCID_{50}$, the designation of the infectious titer of a virus for cell cultures: a minimal dilution of a virus that causes the death of 50% of monolayer cells, expressed as the common logarithm.

Table 3. Antiviral activity of carbocyclic derivatives of dipeptide H-Pro-Trp-OH toward the infection caused by HCV in different schemes of administration

Scheme of administration of compounds	Compound	Indicator of the effect of compound (μg/200 μL)		
		CD_{50}	ID_{50}	ID_{95}
6 h prior to infection of cells	**(VII)**	>10.0	1.2	2.5
	(VIII)	>10.0	2.5	5.0
	(IX)	>10.0	<10.0	10.0
	(X)	>10.0	2.5	10.0
	(XI)	>10.0	2.5	10.0
	(XII)	>10.0	<0.3	0.6
At the instant of infection of cells	**(VII)**	>10.0	5.0	10.0
	(VIII)	>10.0	5.0	10.0
	(IX)	>10.0	5.0	10.0
	(X)	>10.0	<5.0	10.0
	(XI)	>10.0	5.0	10.0
	(XII)	>10.0	0.6	1.2
6 h after the infection of cells	**(VII)**	>10.0	5.0	10.0
	(VIII)	>10.0	>10.0	>10.0
	(IX)	>10.0	>10.0	>10.0
	(X)	>10.0	<5.0	10.0
	(XI)	>10.0	10.0	>10.0
	(XII)	>10.0	1.2	10.0

Designations as in Table 1.

also worthy of notice with the ID_{50} values of 1.2, 5.0, and 5.0 μg/200 μL according to the scheme of administration.

Attempts to improve compound (**XII**) by substituting L-alanine, L-cysteine, or L-lysine for L-tryptophan, as well as the introduction of a spacer of glycine residues (from one to three residues) between the cyclohexyloxycarbonyl residue and the dipeptide H-Pro-Trp-OH, led to the loss of the activity of the compound.

Finally, the antiviral activity of compound (**XII**) was compared with the results obtained earlier for the adamantylethylamide tetrapeptide Boc-Lys(Boc)-Gly_3-OH [15] (Table 4). It follows from the data in Table 4 that the structurally different compounds Boc-Lys(Boc)-Gly_3-Rim and (**XII**) (Figs. 1c and 1d), have similar values of antiviral activity in the schemes of the administration of compounds. According to the results of molecular docking in silico, the molecule of compound (**XII**) (similar to the calculations for Boc-Lys(Boc)-Gly_3-Rim) is bound to several helices of protein p7 in the external space of the conducting channel (Figs. 1a and 1b). Presumably, the presence of carbocyclic compounds in the interhelical space of the channel protein mechanically disturbs the mobility of chains for the opening or closing of the pore, which in turn leads to the disturbance of ion transport. This has yet to be elucidated.

Table 4. Comparison of antiviral activity of compounds of dipeptide Cho-Pro-Trp-OH (**XII**) and Boc-Lys(Boc)-Gly_3-Rim toward the infection caused by HCV in SPEV cells

Compounds and the scheme of administration of compounds into cell culture		Indicator of the activity of compound (μg/200 μL)		
		CD_{50}	ID_{50}	ID_{95}
24 h prior to infection	Boc-Lys(Boc)-Gly_3-Rim	>2.5	0.312	0.5
	(XII)	>2.5	0.312	1.0
At the instant of infection of cells	Boc-Lys(Boc)-Gly_3-Rim	>2.5	0.156	1.0
	(XII)	>2.5	<0.5	0.5
24 h after infection	Boc-Lys(Boc)-Gly_3-Rim	>5.0	<0.5	0.5
	(XII)	>5.0	<0.5	0.5

Designations as in Table 1.

EXPERIMENTAL

The following reagents were used: racemic Rim (Zhejiang Kangyu Pharmaceutical Co., China); amantadine hydrochloride, TEA, Nor, Nrb, Qln, Ad, Tha, NMM, and *L*-amino acids (Sigma-Aldrich, United States); and IBCF (Fluka, Switzerland). All solvents used for the condensation and removal of protective groups were preliminarily dried and distilled by standard methods. The resulting compounds were identified by TLC on Merck-Kieselgel 60 F (254) plates in systems: methanol–chloroform 13 : 60 (A), *sec*-butanol–3% ammonia 100 : 44 (B), *n*-butanol–acetic acid–water–pyridine 30 : 3 : 12 : 10 (C), which permitted us to ascertain the complete absence of initial reagents in samples being tested. The molecular weight was determined on a Bruker UltraFlex II MALDI-TOF mass spectrometer using the programs flexControl 1.1 and flexAnalys 2.2 for the retrieval and processing of mass spectra. IR spectra were recorded on an InfraLUM FT-10 IR-Fourier spectrometer. Melting temperatures were measured on an SMP20 Stuart Scientific digital apparatus. The specific optical rotation of the resulting compounds was determined under standard conditions on an A1-EPL automatic polarimeter (a 1% solution of a compound in ethyl ether; cuvette length 0.5 dm).

A highly virulent strain of the avian influenza A (H5N1) virus A/duck/Novosibirsk/56/05 (H5N1) was used, which was isolated during the epizootia among domestic poultry in July 2005 in Novosibirsk oblast [16]. Viruses were obtained from the State Collection of Viruses (SCV) at the Ivanovskii Institute of Virology (Gamaleya Research Center for Epidemiology and Microbiology, the Ministry of Public Health of Russia). The virus-containing material represented a liquid collected from A(H5N1)-infected SPEV cell cultures at the peak of the development of cytopathic effects. The infectious titer of viral strains for cell cultures varied from 5.5 to 6.0 $logTCID_{50}$.

The HCV strain D-1 was obtained from the working collection of cytopathogenic HCV strains isolated from hepatitis C patients (SCV, Ivanovskii Institute of Virology, Gamaleya Research Center for Epidemiology and Microbiology, Ministry of Public Health of Russia). The virus-containing material represented a sample of the HCV-containing liquid collected from infected SPEV cultures. The infectious titer of the viral strain for the given cell type was 7 $logTCID_{50}$/mL.

The culture of SPEV cells was grown as a two-day-old monolayer in 48-well plastic plates in medium 199 (production of the Chumakov Research Institute of Poliomyelitis and Viral Encephalites, Russian Academy of Medical Sciences) supplemented with 100 U/mL of penicillin, 100 U/mL of streptomycin, and 10% bovine serum. The growth of culture cells was controlled using a light optical microscope.

In experiments, the Vero-E6 clonal line sensitive to the reproduction of the influenza A/H5N1 virus was used, which is recommended by the WHO as a substrate for the production of cultured inactivated vaccines. Vero-E6 cells were grown at 37°C in an atmosphere of CO_2 to a complete monolayer in 96-well plastic plates using the Eagle's MEM (PanEko, Moscow) supplemented with glutamine and antibiotics (100 U/mL of penicillin and 100 U/mL of streptomycin) and 7% fetal calf serum (PanEko, Moscow) preliminarily heated in a water bath at 56°C for 20 min. The supporting medium after the adsorption of the virus was Eagle's MEM supplemented with glutamine and antibiotics at the same concentrations and 1% fetal calf serum (Sigma, United States). The peptide bond formation was carried out in one stage at the equimolar ratio by the reaction of mixed anhydrides (Scheme 1).

Boc-Met(O_2)-OH (IVa). Boc_2O (1.4 g, 6.42 mmol) was added in three portions under stirring within 1 h at 45°C to a solution of Met(O_2) (1.0 g, 5.5 mmol) and NaOH (0.22 g, 5.5 mmol) in H_2O (5.0 mL) and Bu^t-OH (4.0 mL). After the termination of the reaction, Bu^t-OH was distilled, and the residue was diluted with H_2O 1.5 times, washed with hexane (3 × 15.0 mL), and acidi-

fied with a 1 M $KHSO_4$ solution to pH 3–3.5. The product was isolated by extraction with ethyl acetate (4 × 15.0 mL). The extract was dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo. The remaining oil spontaneously crystallized. Yield: 1.37 g (88%); mp 112°C; R_f 0.55 (A), 0.81 (B), 0.78 (C); $[\alpha]_D^{20}$ +8°.

Boc-Met(O_2)-Rim (IV). NMM (0.40 mL, 3.5 mmol) was added to compound (**IVa**) (1.0 g, 3.5 mmol) in a mixture (25.0 mL) of $CHCl_3$ and THF (1 : 1.5) after which IBCF (0.47 mL, 3.5 mmol) was added under stirring at –25°C. The mixture was stirred for 10 min, and a solution of Rim (0.77 g, 3.5 mmol) in $CHCl_3$ (10.0 mL), cooled to –20°C, and NMM (0.40 mL, 3.5 mmol) were added. The mixture was stirred for 30 min at –20°C, for 1 h at 0°C, and for 10 h at 20°C. The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate (35.0 mL) and H_2O (10.0 mL) and washed successively with 0.25 M H_2SO_4 (4.0 mL), 0.25 M $KHCO_3$ (2 × 10.0 mL), and H_2O (5.0 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. Ethyl acetate was removed in vacuo to obtain an oily product. Yield: 1.31 g (83%); R_f 0.90 (A), 0.89 (B), 0.91 (C); $[\alpha]_D^{20}$ +7°; IR: ν(NH) at 3356 cm^{-1}; ν(C=O) 1668 cm^{-1}; *m/z*: found $[M + Na]^+$: 465.22; $[M + K]^+$: 481.185; calculated $M(C_{22}H_{38}N_2O_5S)$ 442.61.

HCl*H-Met(O_2)-Rim (III). To a solution of compound (**IV**) (1.0 g, 2.26 mmol) in ethyl acetate (10.0 mL), 4 H HCl (4.4 mL) in ethyl acetate was added at 5°C. The reaction mixture was allowed to stand for 1 h at 20°C with stirring at regular intervals. The product was precipitated and washed with ether. The residue was dried in vacuo. Upon grinding in ether, the remaining oil crystallized. Yield: 0.85 g (96%); mp 138–140°C; R_f 0.53 (A), 0.63 (B); $[\alpha]_D^{20}$ +6°; *m/z*: found $[M + Na]^+$: 443.25; calculated $M(C_{22}H_{38}N_2O_5S)$ 442.61.

TEA-Amt (I) was obtained in a similar way as (**IV**), starting from TEA and amantadine hydrochloride. Yield: 1.68 g (90%); R_f 0.90 (A), 0.81 (B), 0.93 (C); IR: ν(NH) at 3304 cm^{-1}; ν(C=O) 1652 cm^{-1}; *m/z*: found $[M + H]^+$: 288.15; calculated $M(C_{17}H_{21}NOS)$ 287.42 Da.

TEA-Rim (II) was obtained in a similar way as (**IV**), starting from TEA and Rim. Yield: 1.98 g (97%); R_f 0.90 (A), 0.83 (B), 0.92 (C); IR: ν(NH) at 3304 cm^{-1}; ν(C=O) 1652 cm^{-1}; *m/z*: found $[M + H]^+$: 316.19; calculated $M(C_{20}H_{29}NOS)$ 315.47 Da.

Z-Trp-Rim (V) was obtained in a similar way as (**IV**), starting from Z-Trp-OH and Rim. Yield: 1.31 g (83%); R_f 0.85 (A), 0.93 (B), 0.69 (C); $[\alpha]_D^{20}$ +5°; IR: ν(NH) at 3388 cm^{-1}; ν(C=O) 1668 cm^{-1}; *m/z*: found $[M + Na]^+$: 522.34; calculated $M(C_{31}H_{37}N_3O_3)$ 499.64 Da.

Qln-Ala-Pro-Rim (VI) was obtained similarly to (**IV**), starting from Qln-Ala-Pro-OH and Rim. Yield: 0.66 g. (90.4%); mp 145–147°C; R_f 0.81 (A), 0.92 (B), 0.88 (C); $[\alpha]_D^{20}$ +16°; IR: ν(NH) 3386, 3290 cm^{-1}; ν(CH) CH_3-groups 2974, 2900, 2850 cm^{-1}; ν(C=O) 1675, 1628 cm^{-1}; ν(C=C) 1620–700 cm^{-1}; *m/z*: found $[M + H]^+$: 503.33; calculated $M(C_{30}H_{38}N_4O_3)$ 502.64 Da.

Ad-Pro-Trp-OH (VII). To a solution of Ad-Pro-Trp-OMe (0.34 g, 0.69 mmol) in acetone (10.0 mL), 0.25 M NaOH (16.0 mL, 4.0 mmol) was added. The mixture was allowed to stand for 45 min at 20°C. Acetone was removed in vacuo; the aqueous fraction was acidified with 10% citric acid to pH 4 after which the product was extracted with ethyl acetate (20.0 mL) and washed with water (2 × 4 mL). The product was dried over anhydrous Na_2SO_4. Ethyl acetate was removed in vacuo until an oily product formed. Yield: 0.33 g (97%), R_f 0.59 (A), 0.47 (B), 0.59 (C); $[\alpha]_D^{20}$ –23°; IR: ν(NH) at 3298 cm^{-1}; ν(C=O) 1708, 1663 and 1618 cm^{-1}; *m/z*: found $[M + H]^+$: 464.25; calculated $M(C_{27}H_{33}N_3O_4)$ 463.56 Da.

Compounds (**VII**)–(**XI**) were obtained by the method of mixed anhydrides in a similar way as (**IV**), starting from the corresponding carbocyclic acids and HCl*H-Pro-Trp-OMe (**XIV**) followed by the saponification with the ether group, as it was shown for (**VII**) (Scheme 2).

Nor-Pro-Trp-OH (VIII). Yield: 0.29 g (83%); R_f 0.42 (A), 0.47 (B), 0.51 (C); $[\alpha]_D^{20}$ –8°; IR: ν(NH) at 3368 and 3324 cm^{-1}; ν(C=O) 1725, 1657, and 1619 cm^{-1}; *m/z*: found $[M + H]^+$: 422.20; calculated $M(C_{24}H_{27}N_3O_4)$ 421.48 Da.

Nrb-Pro-Trp-OH (IX). Yield: 0.36 g (73%); R_f 0.45 (A), 0.53 (B), 0.53 (C); $[\alpha]_D^{20}$ –8°; IR: ν(NH) at 3305 cm^{-1}; ν(C=O) 1707, 1652, and 1620 cm^{-1}; *m/z*: found $[M + H]^+$: 424.22; calculated $M(C_{24}H_{29}N_3O_4)$ 423.50 Da.

Qln-Pro-Trp-OH (X). Yield: 0.25 g (47%); R_f 0.57 (A), 0.68 (B), 0.57 (C); $[\alpha]_D^{20}$ –36°; IR: ν(NH) at 3288 cm^{-1}; ν(C=O) 1728, 1663, 1617 cm^{-1}; *m/z*: found $[M + H]^+$: 457.19; calculated $M(C_{26}H_{24}N_4O_4)$ 456.49 Da.

Tha-Pro-Trp-OH (XI). Yield: 0.28 g (93%); R_f 0.42 (A), 0.48 (B), 0.51 (C); $[\alpha]_D^{20}$ –30°; IR: ν(NH) at 3368 and 3324 cm^{-1}; ν(C=O) 1726, 1659, and 1618 cm^{-1}; *m/z*: found $[M + H]^+$: 394.17; calculated $M(C_{22}H_{25}N_3O_4)$ 399.43 Da.

Cho-Pro-Trp-OH (XII) was synthesized similarly to (**IV**), starting from Cho-Pro-OH, which was obtained as described in [17], and HCl*H-Trp-OMe followed by the saponification as described for (**VII**). Yield: 1.05 g (91%); R_f 0.80 (A), 0.58 (B), 0.60 (C); $[\alpha]_D^{20}$ –32°; IR: ν(NH) at 3312 cm^{-1}; ν(C=O) 1739 and

1675 cm^{-1}; *m/z*: found $[M + H]^+$: 428.21; calculated $M(C_{23}H_{29}N_3O_5)$ 427.49 Da.

Boc-Pro-Trp-OMe (XIII) was synthesized similarly to (**IV**), starting from Boc-Pro-OH and HCl*H-Trp-OMe. Yield: 4.58 g (95%); R_f 0.89 (A), 0.88 (B), 0.84 (C); $[\alpha]_D^{20}$ −41°; IR: ν(NH) at 3397 and 3143 cm^{-1}; ν(C=O) 1729, 1679 cm^{-1}; *m/z*: found $[M + H]^+$: 417.16; calculated $M(C_{22}H_{29}N_3O_5)$ 415.48 Da.

HCl*H-Pro-Trp-OMe (XIV) was synthesized similarly to (**III**). Yield: 0.21 g (96%); R_f 0.46 (A), 0.49 (B), 0.57 (C); $[\alpha]_D^{20}$ −36°; IR: ν(NH) at 3374 and 3196 cm^{-1}; ν(C=O) 1741, 1679 cm^{-1}; *m/z*: found $[M + H]^+$: 316.16; calculated $M(C_{17}H_{21}N_3O_3)$ 315.37 Da.

The antiviral activity of compounds. The antiviral activity of the compounds synthesized was studied using three schemes of the administration of compounds into a cell culture: 6 h prior to the infection of cells, at the time of infection, and 6 h after the infection of Vero-6 and SPEV cells. The multiplicity of the infection of cells with the influenza A virus and HCV was 0.1–0.01 $TCID_{50}$/cell. The compounds at concentrations 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, and 0.8 μg/mL were added to the wells of 96-well plates (25 μL to each well) with a monolayer of Vero-6 or SPEV cells and supporting medium (200 μL). The cell cultures infected with a virus and treated with the compounds were incubated at 37°C in a CO_2 incubator. After 72 h of incubation, the cell monolayer was stained with methylene blue, the percent of viable cells was determined, and the cytotoxic effect (CD_{50}, a minimal concentration of a preparation that induces the death of 50% of monolayer cells) was estimated using a cytometer (Invitrogen). In the second part of the experiment, by day 6 after the infection, when the complete destruction of monolayer cells in control wells occurred, the inhibitory dose (ID_{50}), a concentration of compounds capable of protecting 50% of cells against the cytopathic action of the virus, and ID_{95}, a minimal concentration of compounds capable of protecting 95% and more of monolayer cells was determined.

Determination of the virucidal activity of compounds. Solutions of compounds at a concentration of 0.1 μg/mL in saline were used in experiments. A solution of the compound was mixed with the virus as follows. A virus-containing material (100 μL) at the initial concentration (8.0 $\log TCID_{50/0.2}$) was added to a solution of a compound (200 μL). The exposure to the virus-containing material was carried out at 24°C for 20 min after which the residual virus infectivity was titrated in each variant of the experiment with SPEV cells. From the difference of titers in the control and experiment, the virucidal activity of a compound, i.e., its capacity to suppress the infectivity of the A/duck/Novosibirsk/56/05 (H5N1) or HVC viruses was estimated.

Thus, the results obtained can be used for other viroporins, such as Vpu of HIV-1, pore-forming proteins 2B of the poliomyelitis virus and 6K of the alphavirus, protein E of coronaroviruses, E5 of the papilloma virus and viroporin of bovine diarrhea virus (BVDV), and other viral ion channels.

REFERENCES

1. Gonzalez, M.E. and Carrasco, L., *FEBS Lett.*, 2003, vol. 552, pp. 28–34.
2. Ewart, G.D., Sutherland, T., Gage, P.W., and Cox, G.B., *J. Virol.*, 1996, vol. 70, pp. 7108–7115.
3. Premkumar, A., Wilson, L., Ewart, G.D., and Gage, P.W., *FEBS Lett.*, 2004, vol. 557, pp. 99–103.
4. Gazzarrini, S., Kang, M., Abenavoli, A., Romani, G., Olivari, C., Gaslini, D., Ferrara, G., Etten, J.L., Kreim, M., Kast, S.M., Thiel, G., and Moroni, A., *Biochem. J.*, 2009, vol. 420, pp. 295–303.
5. Miura, T. and Takeuchi, H., *Biochemistry*, 2001, vol. 40, no. 20, pp. 6053–6060.
6. Oxford, J.S. and Galbraith, A., *Pharmacol. Ther.*, 1980, vol. 11, pp. 181–262.
7. Hay, A.J., Wolstenholme, A.J., Skehel, J.J., and Smith, M.H., *EMBO J.*, 1985, vol. 4, pp. 3021–3024.
8. Kendal, A.P. and Klenk, H.D., *Arch. Virol.*, 1991, vol. 119, pp. 265–273.
9. Griffin, S.D., Beales, L.P., Clarke, D.S., Worsfold, O., Evans, S.D., Jaeger, J., Harris, M.P., and Rowlands, D.J., *FEBS Lett.*, 2003, vol. 535, pp. 34–38.
10. Griffin, S., Stgelais, C., Owsianka, A.M., Patel, A.H., Rowlands, D., and Harris, M., *Hepatology,* 2008, vol. 48, pp. 1779–1790.
11. Lin, C., Lindenbach, B.D., and Pragai, B.M., *J. Virol.*, 1994, vol. 68, pp. 5063–5073.
12. Griffin, S.D., *Proc. Natl. Acad. Sci. U. S. A.*, 2009, vol. 106, pp. 12567–12568.
13. Shibnev, V.A., Garaev, T.M., Finogenova, M.P., Shevchenko, E.S., and Burtseva, E.I., *Khim. Farm. Zh.*, 2012, vol. 46, no. 1, pp. 3–4.
14. Shibnev, V.A., Garaev, T.M., Deryabin, P.G., Finogenova, M.P., Botikov, A.G., and Mishin, D.V., *Byull. Eksp. Biol. Med.*, 2016, vol. 161, no. 2, pp. 247–251.
15. Shibnev, V.A., Deryabin, P.G., Finogenova, M.P., Garaev, T.M., and Mishin, D.V., RF Patent no. RU 2524216 C1, 2014.
16. L'vov, D.K., Shchelkanov, M.Yu., Deryabin, P.G., Grebennikova, T.V., and Prilipov, A.G., RF Patent no. RU 2309983 C1, 2005.
17. Poroshin, K.T., Shibnev, V.A., and Grechishko, V.S., Izv. Akad. Nauk, Ser. Khim., 1956, no. 7, pp. 1294–1295.

Translated by S. Sidorova