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SEARCH FOR NEW DRUGS

SOME PATHWAYS TO OVERCOMING DRUG RESISTANCE OF INFLUENZA A VIRUS TO ADAMANTANE DERIVATIVES

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A series of adamantane derivatives incorporating amino-acid residues have been synthesized for the first time and their antiviral activity with respect to influenza A virus subtypes A(H1N1)v and A(H3N2) has been studied. It is established that some of the synthesized compounds can inhibit influenza A virus strains resistant to rimantadine. Thus, restoration of the antiviral activity of rimantadine with respect to resistant influenza strains is possible.

Key words: adamantane derivatives, amino acids, influenza A, rimantadine, drug resistance, 1-adamantanecarboxylic acid.

The biological activity of adamantane derivatives is due to the symmetry and steric bulkiness of the structure and the significant lipophilicity of the rigid hydrocarbon framework. This enables them to penetrate easily through biological membranes. Therefore, modification of organic compounds by an adamantyl radical changes significantly their biological activity, often enhancing it.

1-Aminoadamantane (midantane) hydrochloride was the first derivative used in medical practice. It exhibits antiviral activity against influenza A strains. Rimantadine was discovered in the 1960s and has been used since the start of the 1980s to treat and prevent influenza infection. Both drugs affect the proton conduction channel (M2) of influenza A virus. The drugs are ineffective against influenza B virus.

A large number of strains that are completely resistant to midantane and rimantadine are currently known. The number of resistant influenza strains increases every year because of spontaneous mutations in the virus genome. This necessitates expanded research on the reasons for the development of resistance and ways of overcoming it by creating new antiviral drugs [1].

One approach to "resuscitating" the antiviral properties of this class of compounds is to incorporate into them additional active functional groups that can disrupt proton transport through the virus membrane by interacting with the transmembrane domain. A source of such active functional groups could be amino acids and peptides incorporated into midantane and rimantadine using peptide synthesis techniques.

We synthesized amino-acid and peptide derivatives of 1-adamantane carboxylic acid and rimantadine and studied their activities against influenza A viruses (H1N1v and H3N2). It was found that several of the resulting adamantane derivatives inhibited influenza A virus strains that were resistant to rimantadine. Thus, it was shown that the antiviral properties of rimantadine could be resuscitated.

The peptide bond between the carbocycle containing the amine and amino acids protected at the amine by *tert*-butyloxycarbonyl (Boc-) was formed in one step using the reaction of mixed anhydrides in equimolar amounts (Scheme 1).

Derivatives of 1-adamantane carboxylic acid and 1,3-adamantane diacetic acid were also synthesized by the mixed anhydride method. The mixed anhydride of adamantane carboxylic acid reacted with esters of the corresponding amino acids.

EXPERIMENTAL CHEMICAL PART

We used rimantadine (Zhejiang Kangyu Pharmaceutical Co., China) and L-amino acids (Nova Biochem). The products were identified using TLC on Silufol and Dc-Kieselgel 60 (Merck) plates and solvent systems *sec*-BuOH:NH_4OH

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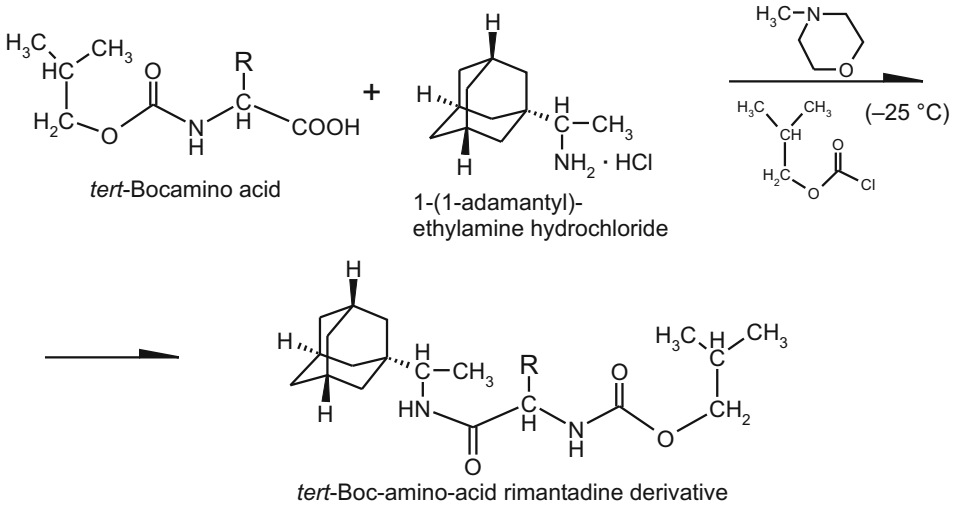
Scheme



General scheme for preparing amino-acid rimantadine derivatives. R is the amino-acid functional group.

(3%) (100:44) (A), MeOH:$CHCl_3$ (13:60) (B), and BuOH:HOAc:H_2O:Py (30:3:12:10) (C). This and mass spectrometry proved that traces of rimantadine, amantadine, and 1-adamantane carboxylic acid were not present in the tested samples. An automated polarimeter (A1-EPL) was used for polarimetric determinations. Melting points were determined on a VEB Analytik Boetius apparatus. Elemental analyses for C, H, and N agreed with the empirical formulas. Amino acids in the products were identified by acid hydrolysis in HCl (6 N) at 105°C for 12 h with identification of the free amino acids by TLC using phenol:H_2O (50:50) and *n*-BuOH:HOAc:H_2O (3:2:2).

***tert*-Butyloxycarbonylsarcosyl-1-adamantylethylamine (Boc-Sar-Rim, II).**

Boc-Sar-OH (1.5 g, 0.0079 mol) in $CHCl_3$ (15 mL) was treated with N-methylmorpholine (NMM, 0.87 mL, 0.0079 mol), cooled to (–20) – (–25)°C, stirred, treated with *iso*-butylchloroformate (1.08 mL, 0.0079 mol), treated with the second component 1-(1-adamantyl)ethylamine (1.71 g, 0.0079 mol) prepared beforehand and cooled to –20°C in $CHCl_3$ (15 mL) with NMM (0.87 mL, 0.0079 mol), and stirred for 30 min, then for 1 h at 0°C, and then for 2 h at 24°C.

The solvents were vacuum distilled in a rotary evaporator at 10 mm Hg. The solid was dissolved in EtOAc (35 mL) and extracted successively with H_2O (5 mL), $NaHCO_3$ solution (0.5 N, 10 mL, 2×), citric acid solution (10%, 4 mL), and again with H_2O (5 mL). The organic layer was dried over anhydrous Na_2SO_4. The EtOAc was vacuum distilled to afford white crystals. Yield 2.55 g (83%), $R_f^A = 0.86$, $R_f^B = 0.68$, $R_f^C = 0.91$, $[\alpha]_D^{20} = -2.5°$, mp 115 – 117°C.

If necessary, the *tert*-butyloxycarbonyl group was removed by EtOAc saturated with HCl (4 N) at 20 – 24°C for 30 min.

The other amino-acid rimantadine derivatives were prepared analogously. These included *tert*-butyloxycarbonylornithyl-1-adamantylethylamine (**I**), citrullyl-1-adamantylethylamine (**III**), *tert*-butyloxycarbonylcitrullyl-1-adamantylethylamine (**IV**), α-di-*tert*-butyloxycarbonyllysyl-ε-*N*-carbobenzoxylysyl-1-adamantylethylamine (**V**), 1-adamantylethylamide of 4-*N*-*tert*-butyloxycarbonylaminobutanoic acid (**VI**), 1-adamantylethylamide of α-lipoic acid (**VII**), histidyl-1-adamantylethylamine (**XI**), tyrosyl-1-adamantylethylamine (**XII**), 1-adamantylethylamide of 2-*N*-*tert*-butyloxycarbonylethanesulfonic acid (*tert*-butyloxycarbonyltauryl-1-adamantylethylamine, **XIV**), 1-adamantylethylamide of 2-aminoethanesulfonic acid (tauryl-1-adamantylethylamine, **XV**), *tert*-butyloxycarbonylhistidyl-adamantylamine (**XVI**), and histidyladamantylamine (**XVII**).

Methyl ester of α-*tert*-butloxycarbonyl-ε-*N*-adamantyllysine [Boc-Lys(Ad)-OCH_3, IX]

A solution of adamantane carboxylic acid (0.9 g, 0.005 mol) and NMM (0.55 mL, 0.005 mol) in $CHCl_3$ (10 mL) was cooled to –20°C, stirred, treated with *iso*-butylchloroformate (0.68 mL, 0.005 mol), stored for 10 min, treated with a previously cooled solution of Boc-Lys(NH_2)-OCH_3 (1.3 g, 0.005 mol) in $CHCl_3$ (10 mL), and stirred for 30 min at –20°C, 1 h at 0°C, and 2 h at 24°C. The $CHCl_3$ was vacuum distilled. The solid was dissolved in EtOAc (20 mL) and extracted successively with H_2O (5 mL), $NaHCO_3$ solution (0.5 N, 10 mL, 2×), citric acid solution (10%, 4 mL), and H_2O (5 mL). The organic layer was dried over anhydrous Na_2SO_4. The EtOAc was vacuum distilled to afford an oily product that was dried at 1 mm Hg. Yield 2.1 g (quantitative, oil), $R_f^A = 0.81$, $R_f^B = 0.85$, $R_f^C = 0.88$, $[\alpha]_D^{20} = -10°$. The Boc group in the resulting lysine derivatives was removed in HCl (4 N) as described above.

Compounds with α-*tert*-butyloxycarbonyl-ε-*N*-adamantyllysine (**VIII**), 1,6-diaminoaminohexane-1-amideadamantane carboxylic acid (**X**), and the methyl ester of diseryl-1,3-adamantane diacetic acid (**XIII**) were prepared analogously.

1,3-Adamantane diacetic acid and two equivalents of the corresponding serine methyl ester hydrochloride were used for **XIII**.

Where necessary, the ester of the resulting *N*-adamantylpeptides was removed using NaOH (0.2 N) for 45 min at room temperature (**VIII**).

EXPERIMENTAL BIOLOGICAL PART

Antiviral activity

We used influenza viruses pandemic strain A/IIV-Moscow/01/2009 (H1N1)swl similar to standard strain A/California/7/2009 (H1N1)swl [2] and A/Moscow/26/2009 (H3N2) that were resistant to rimantadine.

Antiviral activity was studied in 96-well plates with a formed monolayer of MDCK cell culture tissue. Rimantadine and the tested compounds (adamantane derivatives) at concentrations of 5.0 μg/mL were added at the same time that the cell monolayer was infected. Plates were incubated for 24 h at 37°C, after which the reaction was stopped by treating cells with acetone (80%) in phosphate buffer. Cell immunoenzyme analysis (IEA) was carried out according to the literature [3, 4]. The percent inhibition of virus activity by the compounds was determined as the ratio [experimental optical density $(OD)_{492}$ minus OD_{492} of a cell control/OD_{492} of a virus control minus OD_{492} of a cell control] multiplied by 100%.

Cytotoxic activity

Toxicity of the compounds was studied by placing them at various concentrations on a monolayer of MDCK cell culture tissue in 96-well plates and incubating at 37°C. The condition of the cell monolayer was checked under a microscope. The concentration of the compound causing degeneration of 50% of the cells compared with a control was taken as the mean toxic concentration (CT_{50}). The lowest concentration of a compound causing degeneration of cells was considered the minimal toxic concentration (MTC). The maximum tolerated dose (MTD) was considered half the concentration of a compound that did not have a toxic effect on the cells. Cytotoxicity was estimated by a colorimetric method. After incubation for 72 h at 37°C, the monolayer was rinsed with PBS solution. The number of viable cells was determined on an automated spectrophotometer at wavelength 450 nm by comparing the intensity of the solution color in control and test wells after adding neutral red. The concen-

TABLE 1. Physicochemical Constants of Adamantane Derivatives

	Compound	Yield, g	mp, °C	$[\alpha]_D^{20}$	TLC R_f		
					A	B	C
I	$(Boc)_2$-Orn-Rim	0.73 (91 %)	140 – 142	– 3.5	0.64	0.88	0.94
II	Boc-Sar-Rim	2.55 (83 %)	115 – 117	– 2.5	0.68	0.86	0.91
III	H-Cyt-Rim	0.36 (97 %)	oil	– 2 (acet)	does not move	0.48	0.57
IV	$(Boc)_2$-Cyt-Rim	0.45 (89 %)	oil	– 2.5	0.81	0.82	0.93
V	$(Boc)_2$-Lys-Lys(Z)-Rim	0.83 (83 %)	oil	– 20	0.88	0.87	0.87
VI	Boc-GABA-Rim	3.62 (quant.)	106	0	0.70	0.51	0.64
VII	TOA-Rim	0.92 (81 %)	114	- (racemate)	0.87	0.86	0.89
VII	NH_2-Lys(Ad)-OH	0.75 (87 %)	amorph.	– 4	0.15	0.27	0.58
IX	NH_2-Lys(Ad)-OMe	1.09 (83 %)	amorph.	– 4	0.30	0.24	0.53
X	Ad-HDA	0.23 (93 %)	95	0	0.80	0.89	0.93
XI	H-His-Rim	0.98 (86 %)	210	– 6	does not move	0.50	0.75
XII	H-Tyr(OH)-Rim	0.25 (90 %)	185	– 16	0.14	0.74	0.9
XIII	Ad-$(CH_2$-Ser(OMe))$_2$	0.23 (70 %)	amorph.	+ 8	0.26	0.76	0.75
XIV	Boc-Tau-Rim	0.43 (55 %)	122	+ 10	0.45	0.54	0.95
XV	H-Tau-Rim	0.33 (97 %)	213 (dec)	0	does not move	0.37	0.70
XVI	Boc-His-AmAd	0.73 (60%)	amorph.	– 3	0.88	0.85	0.73
XVII	H-His-AmAd	0.45 (92 %)	oil	– 6	does not move	0.33	0.51
	Rimantadine		< 300	- (racemate)	0.51	0.46	0.70

Note: Rim, rimantadine molecular framework [1-(1-adamantyl)-aminoethane]; AmAd, midantane molecular framework (1-aminoadamantane); Ad, 1-adamantane carboxylic acid bonded to the amino-acid amine; Cit, citrulline moiety; GABA, γ-aminobutyric acid; TOA, thioctic (lipoic) acid; HDA, 1,6-hexanediamine; Tau, taurine sulfonylamino acid; $[\alpha]_D^{20}$, refractive index in EtOH for c = 1 μg/mL except for **III** that was dissolved in HOAc.

tration of a compound that inhibited the OD by 50% compared with a cell control was taken as the 50% cytotoxic dose (CD_{50}).

RESULTS AND DISCUSSION

Table 2 presents test results for the ability of several of the prepared compounds to inhibit viruses A(H1N1)v and A(H2N3) and the cytotoxic activity of the compounds on MDCK cell culture.

Antiviral activity of the compounds was studied at a concentration of 5 μg/mL. The percent inhibition of the compounds given in Table 2 is the arithmetic mean. Only the most interesting results are shown because many compounds turned out to be slightly active and several exhibited toxicity for the cells.

It can be concluded by analyzing the results that the presence of a free amine in the linear molecules did not help to bind the adamantane construction in the pore of the M2-channel of A(H1N1)v and A(H2N3). This was also seen for the aminoadamantanes rimantadine and midantane. However, an inhibiting effect on the virus did appear if the amine was protected by a *tert*-butyloxycarbonyl (Boc-) group. Such compounds were the principal amino acids ornithine (**I**) and sarcosine (**II**). The presence of a highly hydrophobic portion together with the lipophilic carbocyclic adamantane created the optimum structure for penetrating the membrane bilayer of the virus capsule and disrupting replication processes. The citrulline derivative (**III**) behaved analogously except that its antiviral activity was weaker and there was no correlation between the two viruses. The effect was almost minimal on A(H3N2), in contrast with its effect on A(H1N1)v.

Based on the activity of the ornithine derivative, we synthesized the close structural analog Boc-GABA-Rim (**VI**). This molecule represented rimantadine linked to α-*tert*-butyloxycarbonyl γ-aminobutyric acid. Thus, only one amine and one Boc-group were left. The activity of the compound was slightly less than that of **I**.

Lysine in addition to γ-aminobutyric acid can also act as an analog of ornithine. The lysine derivative with rimantadine did not substantially inhibit the viruses either with free amines or blocked ones. Conversely, the use of 1-adamantane carboxylic acid to synthesize Boc-Lys(NH_2)-OMe produced a new steric configuration in these compounds (**VIII** and **IX**). The α-amine of lysine was protected by the Boc-group whereas the ε-amine was available for binding to 1-adamantane carboxylic acid. Saponification of the ester (methoxy) group of lysine (**IX**) had practically no effect on the activity against both strains (**VIII**).

Compound **V** represented rimantadine linked to lysine dipeptide in which the ε-amine of one unit was protected by a carbobenzoxy group and both amines of the second unit were protected by Boc-groups. It inhibited only A(H1N1)v and had an extremely weak effect on A(H3N2).

The molecule containing histidine (**XVI**) exhibited stable suppression of influenza A virus replication.

The histidine derivatives of rimantadine (**XI**) and aminoadamantane (midantane) (**XVI** and **XVII**) exhibited positive effects. This could be explained by the fact that the presence of another (fifth) histidine unit in the channel pore hindered operation of the proton pump, possibly because of competing imidazole ring protonation processes.

The tyrosine derivative (**XII**) induced moderate (30 – 40%) suppression of virus production.

Blocking the tyrosine hydroxyl with a benzyloxy group produced the very active compound H-Tyr(Obzl)-Rim at a concentration of 5 μg/L. However, increasing the concentration of this compound to 40 μg/mL produced a toxic effect.

The most active compound against both virus strains and yet non-toxic was the derivative of 1,3-adamantane diacetic acid with serine methyl ester (**XIII**). The cell culture was not affected by the action of this compound even upon reaching a concentration of 80 μg/mL.

The Boc-protected derivative of the aminosulfonic acid taurine (**XIV**) was slightly active against A(H1N1)v (30 – 41%). However, it inhibited seasonal A(H3N2). On the other hand, taurine with a free amine (**XV**) suppressed development of both virus strains by greater than 70%.

Another S-containing rimantadine derivative that included lipoic acid [3-(4-carboxybutyl)-1, 2-dithiolane,

TABLE 2. Antiviral and Cytopathic Activity of Adamantane Derivatives

	Compound	Activity, %		MTD, μg/mL
		A(H1N1)v	A(H3N2)	
I	Boc-Orn-Rim	71.06	66	40
II	Boc-Sar-Rim	70.8	64	40
III	H-Cyt-Rim	36	n/e *	40
IV	Boc-Cyt-Rim	53	25	> 80
V	$(Boc)_2$-Lys-Lys(Z)-Rim	69	30	> 80
VI	Boc-GABA-Rim	48	52	40
VII	TOA-Rim	78	89	80
VIII	NH_2-Lys(Ad)-OH	54	25	> 80
IX	NH_2-Lys(Ad)-OMe	47	25	> 80
X	Ad-HDA	74	63	> 80
XI	H-His-Rim	91	94	40
XII	H-Tyr(OH)-Rim	29	38	> 80
XIII	Ad-$(CH_2Ser(OMe))_2$	98	90	> 80
XIV	Boc-Tau-Rim	35	69	> 80
XV	H-Tau-Rim	76	72	> 80
XVI	Boc-His-AmAd	98	60	40
XVII	H-His-AmAd	n/e	26.8	40
	Rimantadine	n/e	n/e	40

* n/e, no viral inhibition effect.

thioctic acid, **VII**], a cyclic disulfide, exhibited significant suppression (~80%) against both influenza A virus strains.

Thus, the study of the antiviral properties of amino-acid derivatives of the adamantane series found that introducing functional groups led to a resuscitation of the antiviral properties, i.e., the resistance was overcome.

The adamantane structure acts as a membrane carrier to transport functionally active groups to influenza A virus M2 protein.

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