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SYNTHESIS AND ANTIVIRAL ACTIVITY OF SOME AMINO ACIDS DERIVATIVES OF INFLUENZA VIRUS DRUGS

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Abstract. A series of new rimantadine (RS)-1-(1-adamantyl)ethanamine) analogues with some amino acids (alanine, valine, phenylalanine) have been synthesized and their antiviral activity against influenza A virus has been studied. Among the tested compounds the moderate antiviral activity has been found for the alanyl-rimantadine.

Keywords: *Rimantadine, amino acids, influenza virus*

Introduction

Influenza A viruses are the major human pathogens that cause annual epidemics and occasional pandemics. In the past 100 years, influenza epidemic and pandemics had caused serious impact on global morbidity, mortality and economy. There are two subtypes of influenza A viruses based on the glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are found on the surface of the viral envelope (Chen et al., 2015). Currently, two classes of anti-influenza drugs are found on the market. Oseltamivir and zanamivir are the neuraminidase inhibitors. They block the active site of neuraminidase and allow stopping virus replication. There is another class drugs called M2 ion channel blockers. One of the currently available drugs for the prevention and treatment of influenza virus is amantadine and its analogue rimantadine. They inhibits the M2 proton channel of influenza A virus, yet most of the currently circulating strains of the virus carry mutations in the M2 protein that render the virus amantadine-resistant (Rey-Carrizo et al., 2014). 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 (Shibnev et al., 2012). The clinical usefulness of amantadine and rimantadine is limited due to the increasing incidence of adamantane-resistant viruses in the population (Bright, 2005). Moreover, the M2 ion channel blockers inhibit only influenza A virus replication and are associated with neurological side effects. Therefore we have synthesized and tested for antiviral activity against Influenza A viruses new amino acids derivatives of rimantadine (Fig. 1).

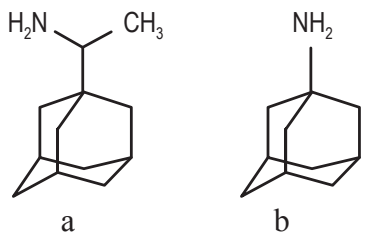


Fig. 1. M2 ion channel blockers - rimantadine (a) and amantadine (b)

Major problems of medicine besides the emergence of resistance it turns out and the potential passage of drug through the cell membrane and transfer it to the target cell (Griffiths & Sjövall, 2010). In order to facilitate the passage of biologically active compounds through the cell membrane is to connecting them with transport molecules. A good approach to solving this problem is modification of biologically active substances with α-amino acids. They are harmless molecules since they build up every known protein of living organisms.

Experimental

The rimandatine substance, amino acids, isobutyl chloroformate and the solvents were purchased from Sigma-Aldrich. Triethylamine (TEA) was purchased from Merck, Germany. TLC analysis was performed on aluminum silica gel sheets 60 F254 plates (Merck) and spots were detected using an UV lamp at 254 nm and/or ninhydrin reagent 2% solution. As eluting systems are used chloroform / methanol (95:5). All solvents were distillated before use.

Synthesis of rimantadine analogues

The *tert*-butyloxycarbonyl (Boc) amino acids (Boc-Ala, Boc- Phe, Boc-Val) (69 mmol) were dissolved in 2 ml CH_2Cl_2. The solution was cooled to -10ºC, added TEA (69 mmol) and dropwise isobutyl chloroformate (69 mmol). The rimantadine (46 mmol)

were dissolved in 2 ml $CHCl_3$, added TEA (46 mmol). After 15 min the solutions were mixed and stirred for 30 min at -10ºC. The reaction mixture was stirred for 1h at 0ºC. The mixture was poured into 5% $NAHCO_3$, extracted with CH_2CI_2, washed with brine, dried over Na_2SO_4 and concentrated *in vacio*. The residues was purified by TLC on Kieselgel $60F_{254}$ using the solvent system chloroform/ methanol (95:5). Boc-Ala-Rim- M.m.= 350. 9; Boc-Val-Rim - M.m.= 378; Boc-Phe-Rim- M.m.= 426.

The resulting white solid, Boc-Phe-rimantadine, Boc-Ala-rimantadine and Boc-Val-rimantadine were dissolved in 2 ml of 50% TFA/ CH_2CI_2 and stirred at 0ºC for 1h to remove the Boc group.

Antiviral activity against influenza virus A(H3N2)

Cells: MDCK

Cytotoxicity assay for determination of the 50 % cytotoxic concentrations (CC_{50}) of test compounds in MDCK (Madin Darby canine kidney cell monolayers).

Virus: *Influenza A/H3N2 strain Aichi*

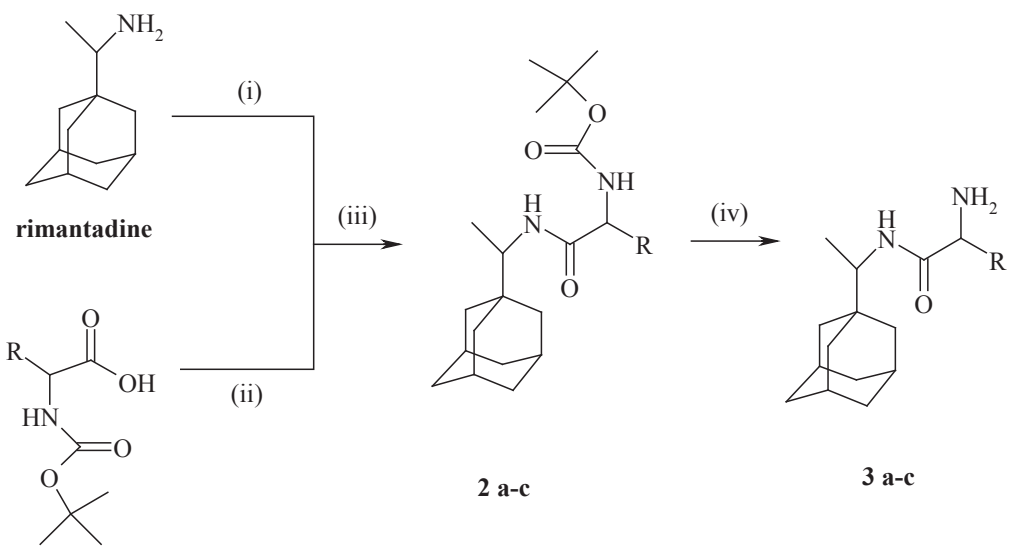
Viral suspension is from the collection of the section "Virology" of the "Stefan Angelov" Institute of Microbiology, Bulgarian Academy of Sciences. The virus is cultured in a maintenance environment DMEM (Dulbecco's Modified Eagles's Medium) (Gibco BRL, USA) with 0.5% fetal veal serum, 10 mM HEPES (Merk, Germany) and antibiotics (penicillin 100 UI / mL and streptomycin 100 μg / mL) at 37 º C in the presence of 5% CO2. After seeding in microtitre plates, MDCK cells were incubated at 5 % CO2, 37 °C and 95 % humidity for 48 h. Thereafter, the cell culture medium was aspirated and serially diluted compound concentrations in fresh cell culture medium were added (100 μl/well; 2 parallels/concentration, dilution factor 2). Six untreated wells were used as cell control (negative control). 72 h after compound addition and incubation cell were stained with a crystal violet/methanol solution. After dissolving away the stain, the optical density (OD) of individual wells was determined in a Dynatech microplate Photometer (550 /630 nm) and compared with the mean optical density of the 6 cell controls.

Results and discussion

The modification of various biological active compounds with amino acids allows them to be hydrolyzed more quickly under the influence of plasma enzymes, thus leading to their transformation as prodrugs. On the other hand these compounds exhibited poor stability in aqueous solution as exemplified with esters of metronidazole, acyclovir, ganciclovir, corticosteroids. To overcome such problem is the use of adequately selected spacer groups (Stankova, 1999).

For our synthesis of amino acid derivatives of rimantadine we use mixed anhydrides peptide synthesis method. The mixed anhydride method of coupling used in peptide synthesis involves reaction of a protected amino or peptide acid with an alkyl chloroformate in the presence of a tertiary amine base to give the mixed carboxylic acid - carbonic acid anhydride. The anhydride is then reacted with an amine nucleophile, which is either an amino acid ester α or a peptide ester, giving the protected peptide (Chen, 1987). The *tert*-butyloxycarbonyl group was removed by TFA at 0ºC.

The synthetic rout for the preparation of amino acids derivatives of rimantadine is shown in Sheme 1. Boc-AA (1a-c) were dissolved in CH_2Cl_2 to -10ºC, added triethylamine (TEA) and isobutyl chloroformate. Rimantadine were dissolved in $CHCl_3$ and the solutions were mixed, stirred to produce the Boc-amino acids analogues (2 a-c). The resulting white solid, Boc-AA were dissolved in TFA to remove the Boc group (3a-c). The ESI-MS analysis proved the identity of the final products **(**Figs. 2-4**).**



(i) Et_3N, $CHCl_3$, -10ºC, (ii) Et_3N, IBCF, CH_2Cl_2, -10ºC, 15min (iii) 1,5h, -10ºC, (iv) TFA/ CH_2Cl_2

R = -CH3 – Alanine (a)

R= $-CH(CH_3)_2$ – Valine (b)

R= $-CH_2-C_6H_5$ – Phenylalanine (c)

Sheme 1. Synthesis of rimandatine analogues with amino acids

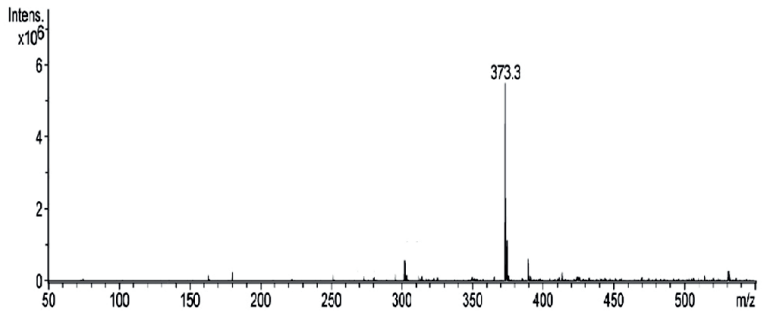


Fig. 2. ESI-mass spectrum of *tert*-butyloxycarbonyl-alanyl-rimantadine. Mass-spectrum of Boc-Ala-Rim: 373.3 $[M + Na]^+$

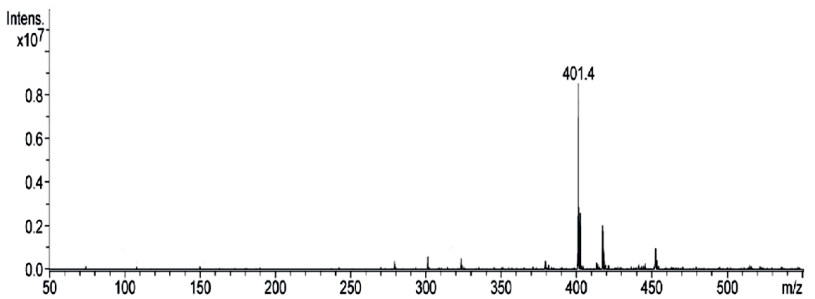


Fig. 3. Mass spectrum of *tert*-butyloxycarbonyl-valyl-rimantadine. Mass-spectrum of Boc-Val-Rim: 401.4 $[M + Na]^+$

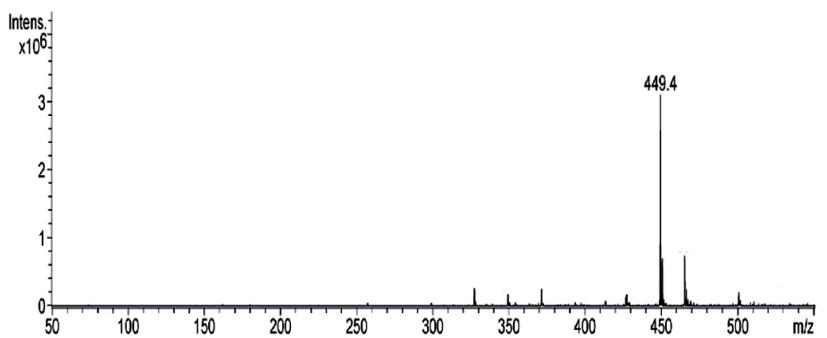


Fig. 4. Mass spectrum of *tert*-butyloxycarbonyl-phenylalanyl-rimantadine. Mass-spectrum of Boc-Phe-Rim: 449.4 $[M + Na]^+$

Biological activity

Initially the new analogues **3a-c** were evaluated for their antiviral activity towards influenza virus *A(H3N2)*. Results of the antiviral screening of the amino acids analogues of rimantadine are shown in Table 1. Analogues of valine (**3b)** and phenylalanine (**3c**) with rimantadine do not show activity, and analogue of alanine exhibited a moderate activity.

Table 1. Antiviral activity and cytotoxicity of rimantadine derivatives

Compounds	**Cytotoxicity (CC_{50})**	**IC_{50}**
Ala-Rim (**3 a**)	200	39,45
Val-Rim (**3 b**)	205.82	0
Phe-Rim (**3 c**)	20.30	0

These results demonstrate that modification of rimantadine with aliphatic and aromatic amino acids does not lead to an antiviral effect compared to amino acids with guanidino group.

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