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Amino acids amides of anti-influenza drugs: synthesis and biological activities

B. Stoykova[1], M. Chochkova[1*], L. Georgiev[1], G. Ivanova[2], L. Mukova[3], N. Nikolova[3], L. Nikolaeva-Glomb[3], Ts. Milkova[1], M. Štícha[4]

[1] *Department of Chemistry, South-West University "Neofit Rilski", 2700 Blagoevgrad, Bulgaria*
[2] *Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, 4169-007 Porto, Portugal*
[3] *The Stephan Angeloff Institute of Microbiology Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[4] *Department of Organic Chemistry, Faculty of Science, Charles University, 12843 Prague 2, Czech Republic*

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Influenza A viruses are amongst the most severe human pathogens leading to high morbidity and mortality worldwide. Due to the high mutation rate and the unpredictable potential for influenza pandemic outbreaks, the development of novel anti-influenza drugs is an undeniably attractive area of research.

In the present study amino acid amides of rimantadine and oseltamivir were synthesized and their *in vitro* antiviral activity against influenza A viruses (A/H3N2) was studied. Results revealed that amide modification of N_{α}- and side chain protected tyrosine, histidine, aspartic- and glutamic acids did not exhibit significant enhancement of the *in vitro* effect against influenza A virus strain.

Keywords: amino acids amides, aminoadamantanes, influenza virus neuraminidase inhibitors

INTRODUCTION

Influenza viruses are RNA viruses responsible for an acute infectious disease, commonly known as a flu. Those pathogens belong to *Orthomyxoviridae* family and they are classified into three types (A, B and C) on the basis of differences in their nucleoprotein antigens [1, 2]. Unlike the antigenic stability of influenza type C, the genetic variation of hemagglutinin (HA) and neuraminidase (NA) antigens in types A and B leads to a frequent occurrence of viral mutations through the mechanisms of antigenic drift and shift. Thus, a rise to a rapid development of new virus trains is given which could be a serious threat to the human population [3, 4].

In recorded world history influenza infection has generated some of the worst pandemics. The 1918-1919, influenza pandemic (Spanish flu) swept across the world in three waves and was responsible directly or indirectly for over 20 million deaths-more than doubling the total casualty of the previous leader, the Black Death [5]. Since then, at least 3 pandemics and numerous milder localized influenza epidemics have been recorded.

At the present time, influenza continues to be a serious threat to human health. Affecting the population irrespective of age, it causes tremendous economic losses and also poses a global concern due to its unpredictable, pandemic potential and pathogenesis.

Although vaccination is the mainstay of influenza prophylactic treatment, this primary prevention strategy is associated with significant drawbacks. For instance, annual update is required due to the widely varying virus prevalence between years. Moreover, vaccines and circulating virus strains must be closely matched, and there have been well recognized vaccine failures [6].

Therefore, effective antiviral agents are of utmost importance for influenza treatment [7]. Although two clinically relevant classes of anti-influenza compounds are available, the effectiveness of the neuraminidase inhibitors (oseltamivir, zanamivir) is preferable because of the high level of resistance to the amantadine (amantadine, rimantadine) observed worldwide [7, 8].

A promising approach for "resuscitation" the antiviral properties of M2 ion channel blockers would be the modification of the structure of the antiviral compound by incorporating additional active functional groups. The main goal is disruption of the proton transport through the virus membrane via interacting with the transmembrane domain. A source of such active functional groups could be amino acids and peptides, which can finally play key role as inhibitors of enzymes included in different diseases [9]. The use of amino acid scaffolds as building blocks during drug discovery [10] and the unusual role of amino acids

* To whom all correspondence should be sent:
E-mail: mayabg2002@yahoo.com

as reactants for important drug-like compounds [11] (e.g., benzodiazepines) are potentially relevant for a wide number of applications in the medicinal chemistry. The conserved backbones and variable side chains of amino acids along with their high bioavailability, make them readily enter in biochemical reactions.

Recently *Shibnev V. A. et. al.* reported for several adamantane derivatives with amino acid residues which inhibit resistant to rimantadine influenza A virus strains [12]. Subsequently, in order to investigate the antiviral activity of similar compounds, we modified anti-influenza agents (amantadine, rimantadine and oseltamivir) with amino acid residues.

EXPERIMENTAL

General information

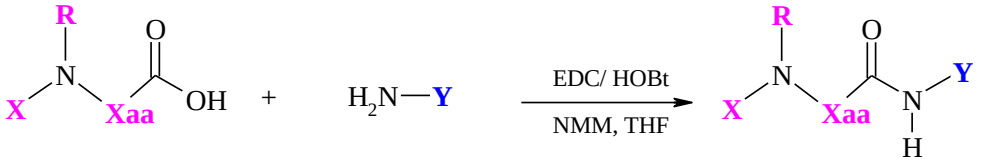
All chemicals were of analytical grade and were purchased from Sigma-Aldrich. Ethyl (3R ,4R, 5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (oseltamivir) was obtained from Aopharm (China).

Melting points were determined using an apparatus „Stuart SMP10“. UV spectra of the amides were measured with an Agilent 8453 UV–Vis spectrophotometer. Attenuated total reflectance infrared spectroscopy (ATR-IR) measurements were performed using Thermo Scientific Nicolet iS10 FT-IR device with ID5 ATR accessory (diamond crystal). ^{1}H NMR and ^{13}C NMR were obtained with Bruker Avance II+ 600 and Bruker Avance III 400. The ESI mass spectra were recorded on an Esquire3000 plus instrument. Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60F254 plates (Merck, Germany). Separation of the compounds by preparative thin layer chromatography with silica gel 60 GF254 (Merck, Bulgaria).

Synthesis of anti-influenza drug derivatives incorporating amino acid residues

The synthesis of the desired molecules is outlined in Scheme 1. The couplings between protected amino acid analogues and anti-influenza drugs were performed with EDC/HOBt in tetrahydrofuran [13,14].

The physico-chemical parameters and the IR, NMR MS spectral data of the compounds **1-8** are as follows:



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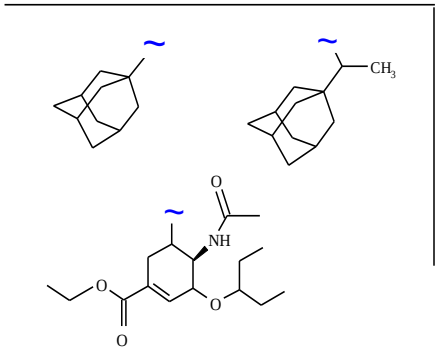

Code	X	R	Xaa	Y
1)	Fmoc-	H	-Tyr(Bu^{t})-	Os
2)	Ac-	H	-Cys-	Os
3)	Boc-	H	-His(Dnp)-	Os
4)	Boc-	CH_3	-Tyr(Bzl)-	Os
5)	Boc-	CH_3	-Tyr(Bzl)-	Am
6)	Boc-	CH_3	-Tyr(Bzl)-	Rim
7)	Z-	H	Glu(OCH_3)-	Rim
8)	Boc-	H	Asp(OBzl)-	Rim

Scheme 1. General scheme for obtaining amino acid derivatives of anti-influenza drugs

N-(9-Fluorenylmethoxycarbonyl)-O-tert-butyl-L-tyrosyl-oseltamivir

(Table 1, Entry 1 (Fmoc-Tyr(Bu^t)-Os)); Yield: 21 %; mp: 226-228°C; UV (C_2H_5OH) λ_{max} = 205, 318 nm; IR (ATR)u_{max}: 3283.1, 2969.2, 2929.6, 1717.4, 1645.8, 1537.4, 1506.5, 1237.9, 737.4; ^{1}H NMR (600 MHz, $CDCl_3$): 0.86 (t, *J*= 7.5 Hz, 3H, -CH_2CH_3), 0.88 (t, *J*= 7.2 Hz, 3H, -CH_2CH_3), 1.23 (s, 9H, -$C(CH_3)_3$), 1.32 (t, 3H, -OCH_2CH_3), 1. 46 (m, 4H, 2 x -CH_2CH_3), 2.02 (s, 3H, -$C(O)CH_3$), 2.31 (m, 1H, =CH-CH_{2a}-), 2.61 (dd, *J*=17.7, 5.0 Hz, 1H, =CH-CH_{2b}-), 2.74 (dd, J=14.4, 11.4 Hz, 1H, Ar-CH_{2a}-), 3.10 (br. d, *J*=14.4 Hz, 1H, Ar-CH_{2b}-), 3.40 (m, 1H, >$CHCH_2CH_3$), 3.81 (ddd, *J*=10.6, 9.6, 8.7 Hz, 1H, $CH_3C(O)NHCH$<), 3.92 (m, 1H, -CH_2CHNH-), 4.08 (t, *J*=8.7 Hz, 1H, -OCH<), 4.19 (q, *J*=7.2 Hz, 2H, -CH_2CH_3), 4.46 (t, 1H, -CH-CH_2-O), 4.92 (ddd, J = 7.4, 5.8, 5.4 Hz, 1H, >N-$CHCH_2$-), 5.04 (d, 2H, -O-CH_2), 6.65 (s, 1H, =CH-), 6.72 (d, *J*=8.5 Hz, 2H, Ar-H), 7.06 (d, 2H, J = 8.5 Hz, Ar-H), 7.66 (1H, -C(O)NH-), 7.54-7.82 (m, 8H, Ar-H), 8.0 (1H, $CH_3C(O)NH$-); ESI-MS: 754.3$[M+H]^+$, 776.4 $[M+Na]^+$.

N-Acetyl-L-cysteinyl-oseltamivir

(Table 1, Entry 2 (Ac-Cys-Os)): Yield: 10 %; mp: 200-202°C; UV (C_2H_5OH) λ_{max} = 206 nm; IR (ATR)u_{max}: 3265.4, 2933.0, 2935.3, 2859.7, 1716.8, 1652.0, 1537.9, 1369.6, 1242.5, 1054.1 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.97 (t, *J*= 7.34 Hz, 3H, -CH_2CH_3), 0.99 (t, *J*= 7.34 Hz, 3H, -CH_2CH_3), 1.39 (m, 3H, -OCH_2CH_3), 1.45 (s, 1H, -SH), 1.47 (m, 4H, 2 x -CH_2CH_3), 1.89 (s, 3H, -$C(O)CH_3$), 2.03 (s, 3H, -$C(O)CH_3$), 2.28-2.56 (m, 2H, =CH-CH_{2a}-), 2.93-3.19 (m, 2H, -CH_2SH), 3.54 (m, 1H, =CH-$CHOCH(CH_2CH_3)_2$), 3.85 (t, 1H, -$OCH(CH_2CH_3)_2$), 3.92 (m, 1H, -$CHNHC(O)$-), 4.06 (m, 1H, -NH-$CHCH_2$-),4.21 (q, *J*=7.2 Hz, 2H, -OCH_2CH_3), 4.82-4.86 (m, 1H, Hα), 6.66 (d, J = 6.2 Hz, 1H, -C(O)NH), 6.77 (d, 1H, =CH-), 7.88 (d, *J*=8.5 Hz, 1H, -C(O)NH-), 7.95 (d, *J*=9.2 Hz, 1H,-C(O)NH-). ESI-MS: 458.2 $[M+H]^+$, 480.1 $[M+Na]^+$.

N_α-tert-Butoxycarbonyl-$N_{(im)}$-(2,4-dinitrophenyl)-L-histidyl-oseltamivir

(Table 1, Entry 3 (Boc-His(Dnp)-Os)): Yield: 14 %; mp:150-153°C; UV (C_2H_5OH) λ_{max} = 207, 265, 300 nm; IR (ATR)u_{max}: 3366.7, 3189.3, 2935.3, 2859.7, 1704.7, 1678.3, 1623.8, 1515.5, 1338.4, 1156.6 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$):: δ 0.87 (t, *J*= 7.5 Hz, 3H, -CH_2CH_3), 0.89 (t, J= 7.2 Hz, 3H, -CH_2CH_3), 1.31 (t, 3H, -OCH_2CH_3), 1.42 (s, 9H, -$C(CH_3)_3$), 1. 46 (m, 4H, 2 x -CH_2CH_3), 2.05 (s, 3H, -$C(O)CH_3$), 2.33 (m, 1H, =CH-CH_{2a}-), 2.59 (dd, *J*=17.8, 5.2 Hz, 1H, =CH-CH_{2b}-), 3.05-3.14 (m, 2H, -CH_2-im), 3.36 (m, 1H, -$CH(CH_2CH_3)_2$), 3.84 (ddd, *J*=10.5, 9.6, 8.6 Hz, 1H, $CH_3C(O)NHCH$<), 3.93 (m, 1H, -CH_2CHNH-), 4.10 (t, *J*=8.6 Hz, 1H, -OCH<), 4.22 (q, *J*=7.2 Hz, 2H, -OCH_2CH_3), 4.62-4.78 (m, 1H, α-CH), 5.73 (d, 1H, J = 8.0 Hz, NH-Boc), 6.69 (s, 1H, =CH-), 6.86 (s, 1H, Ar^{im} -H), 7.63 (s, 1H, Ar^{im} -H), 7.68 (d, 1H, J = 8.8 Hz, Ar^{Dnp}-H), 7.89 (1H, -C(O)NH-), 8.05 (1H, $CH_3C(O)NH$-),8.57 (ddd, J = 8.8, 2.6, 1.6 Hz, 1H, Ar^{Dnp}-H), 8.89 (1H, Ar^{Dnp} -H); ESI-MS: 716.3 $[M+H]^+$, 738.2 $[M+Na]^+$.

O-Benzyl-N_α-tert-butoxycarbonyl-N_α-methyl-L-tyrosyl-oseltamivir

(Table 1, Entry 4 (Boc-N(CH_3)-Tyr(Bzl)-Os)): Yield: 46 %; mp ~ 90-94˚C; UV (C_2H_5OH) λ_{max} = 206, 225, 277 nm; IR (ATR)u_{max}: 3295, 2971, 2933, 2876, 1695, 1651, 1613, 1511 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): 0.77 (t, *J*= 7.5 Hz, 3H, -CH_2CH_3), 0.82 (t, *J*= 7.2 Hz, 3H, -CH_2CH_3), 1.20 (s, 9H, -$C(CH_3)_3$), 1.22 (m, 3H, -OCH_2CH_3), 1. 41 (m, 4H, 2 x -CH_2CH_3), 1.77 (s, 3H, -$C(O)CH_3$), 2.28 (m, 1H, =CCH_{2a}-), 2.56 (dd, *J*=17.7, 5.0 Hz, 1H, =CCH_{2b}-), 2.63 (s, 3H, >NCH_3), 2.74 (dd, J=14.4, 11.4 Hz, 1H, Ar-CH_{2a}-), 3.05 (br. d, *J*=14.4 Hz, 1H, Ar-CH_{2b}-), 3.40 (m, 1H, >$CHCH_2CH_3$), 3.81 (ddd, *J*=10.6, 9.6, 8.7 Hz, 1H, $CH_3C(O)NHCH$<), 3.92 (m, 1H, -CH_2CHNH-), 4.08 (t, *J*=8.7 Hz, 1H, -OCH<), 4.14 (q, *J*=7.2 Hz, 2H, -CH_2CH_3), 4.7 (1H, >N-$CHCH_2$-), 5.04 (s, 2H, -O-CH_2Ar), 6.65 (s, 1H, =CH-), 6.90 (d, *J*=8.1 Hz, 2H, *m*-Ar), 7.11 (d, 2H, *o*-Ar), 7.30 (2H, *m*-Ar), 7.30 (t, *J*=7.2 Hz, 1H, *p*-Ph), 7.37 (t, *J*= 7.2 Hz, 2H, *m*-Ph), 7.41 (d, *J*=7.2 Hz, 2H, *o*-Ar), 7.66 (1H, -C(O)NH-), 7.92 (1H, $CH_3C(O)NH$-); ^{13}C NMR (150 MHz, DMSO-d_6): 9.1 (-CH_2CH_3), 9.4 (-CH_2CH_3), 14.1 (-O-CH_2CH_3), 22.7 (-CH_3), 25.3 (>$CHCH_2CH_3$), 25.8 (>$CHCH_2CH_3$, isomer), 27.7 (3 x -CH_3), 27.9 (3 x -CH_3, isomer), 29.9 (>CH_2), 30.9 (>NCH_3), 33.4 (>CH_2), 47.5 (-HN-CH<), 47.9 (-HN-CH<, isomer), 53.0 (-C(O)HN-CH<), 59.0 (>$NCHCH_2$-), 60.5 (-OCH_2CH_3), 69.1 (-OCH_2Ph), 74.7 (-OCH<), 78.9 (-OCH<), 81.3 (-OCH<, isomer), 114.6 (=CH-, Ar), 127.5 (=CH-, *o*-Ar), 127.7 (=CH-, *p*-Ar), 128.4 (=CH-, *m*-Ar), 129.8 (=CH-, Ar), 137.2 (=CH-), 137.9 (=CH-, isomer), 129.3 (=Cq), 137.7 (=CHC(O)-), 154.6 (-O(O)C-NCH_3), 156.9 (OCq, Ar), 165.5 (155.4 (-O(O)CC=), 169.8 (HNC=O), 170.0 (-NH-C(O)CH_3); ESI-MS: 580.3 $[M+H-Boc+H]^+$, 624.2 $[M+H-56]^+$, 680.4 $[M+H]^+$, 702.4 $[M+Na]^+$.

O-Benzyl- N_α-tert-butoxycarbonyl- N_α-methyl-L-tyrosyl-amantadine

(Table 1, Entry 5 (Boc-N(CH_3)-Tyr(Bzl)-Am)**:** Yield: 52 %; mp ~ 110-114˚C; UV (C_2H_5OH) λ_{max} = 206, 226, 277 nm; IR (ATR)u_{max}: 3368, 2971, 2909, 2847, 1677, 1662, 1512, 1453, 1388, 1363 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): 1.28 (s, 9H, -C(C$\underline{H}_3$)$_3$), 1.60 (6H, 3 x >C$\underline{H}_2$), 1.90 (6H, 3 x >C$\underline{H}_2$), 1.99 (3H, 3 x >C$\underline{H}$-), 2.68 (3H, >NC$\underline{H}_3$), 2.75 (m, 1H, >CHC$\underline{H}_{2a}$-), 2.97 (dd, *J*=14.2, 5.2 Hz, 1H,>CC$\underline{H}_{2b}$-), 4.62 (1H, >NC$\underline{H}$CH$_2$-), 5.04 (s, 2H, -O-C$\underline{H}_2$Ar), 6.90 (2H, *m*-Ar), 7.13 (2H, *o*-Ar), 7.31 (t, *J*=7.2 Hz, 1H, *p*-Ph), 7.37 (t, *J*= 7.2 Hz, 2H, *m*-Ph), 7.41 (d, *J*=7.2 Hz, 2H, *o*-Ar); ^{13}C NMR (150 MHz, DMSO-d_6): 27.9 (3 x -$\underline{C}$H$_3$), 28.8 (3 x >$\underline{C}$H-), 30.3 (>N$\underline{C}$H$_3$), 36.0 (3 x >$\underline{C}$H$_2$), 40.9 (-$\underline{C}$H$_2$CH<), 50.8 (-HN-$\underline{C}$q), 58.4 (>N-$\underline{C}$HCH$_2$-), 60.1 (>N-$\underline{C}$HCH$_2$-, isomer), 69.1 (-O$\underline{C}$H$_2$Ph), 78.7 (-(O)$\underline{C}$(CH$_3$)$_3$), 114.6 (>$\underline{C}$H-, Ar), 127.6 (>$\underline{C}$H-, *o*-Ar), 127.7 (>$\underline{C}$H-, *p*-Ar), 128.3 (>$\underline{C}$H-, *m*-Ar), 129.8 (>$\underline{C}$H-, Ar), 137.2 (=$\underline{C}$H-), 137.9 (=$\underline{C}$H-, isomer), 129.3 (=Cq), 137.7 (=$\underline{C}$HC(O)-), 154.9 (-OO$\underline{C}$N), 156.8 (OCq, Ar), 169.7 (-HN$\underline{C}$=O); ESI-MS: 419.2 $[M+H-Boc+H]^+$, 463.1 $[M+H-56]^+$, 519.2 $[M+H]^+$, 541.2 $[M+Na]^+$.

O-Benzyl- N_α-tert-butoxycarbonyl- N_α-methyl-L-tyrosyl-rimantadine

(Table 1, Entry 6 (Boc-*N*(CH_3)-Tyr(Bzl)-Rim)**:** Yield: 57 %; mp ~ 89-93˚C; UV (C_2H_5OH) λ_{max} = 205, 226, 277 nm; IR (ATR)u_{max}: 3399, 3257, 2974, 2902, 2883, 1678, 1660, 1641, 1509, 1446, 1391, 1364 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): 0.86 (d, 3H, -NHCHC$\underline{H}_3$), 1.30 (s, 9H, -C(C$\underline{H}_3$)$_3$), 1.42 (6H, 3 x >C$\underline{H}_2$), 1.60 (6H, 3 x >C$\underline{H}_2$), 1.89 (3H, 3 x >C$\underline{H}$-), 2.72 (3H, >NC$\underline{H}_3$), 2.82 (m, 1H, >CHC$\underline{H}_{2a}$), 3.00 (m, 1H,=CC$\underline{H}_{2b}$), 3.51 (br. s, 1H, -NHC$\underline{H}$CH$_3$), 4.80 (1H, >NC$\underline{H}$CH$_2$-), 5.04 (s, 2H, -O-C$\underline{H}_2$Ar), 6.90 (2H, *m*-Ar), 7.16 (2H, *o*-Ar), 7.30 (t, *J*=7.0 Hz, 1H, *p*-Ph), 7.37 (t, *J*= 7.0 Hz, 2H, *m*-Ph), 7.41 (d, *J*=7.0 Hz, 2H, *o*-Ar); 150 MHz ^{13}C NMR (150 MHz, DMSO-d_6): 14.0 (-$\underline{C}$H$_3$), 27.7 (3 x -$\underline{C}$H$_3$), 28.0 (3 x >$\underline{C}$H-), 30.2 (>N$\underline{C}$H$_3$), 34.2 (>$\underline{C}$H$_2$), 36.6 (3 x >$\underline{C}$H$_2$), 37.9 (3 x >$\underline{C}$H$_2$), 51.9 (-HN-$\underline{C}$H<), 58.4 (>N-$\underline{C}$HCH$_2$-), 59.0 (>N-$\underline{C}$HCH$_2$-, isomer), 69.1 (-O$\underline{C}$H$_2$Ph), 78.7 (-O$\underline{C}$(CH$_3$)$_3$), 114.6 (>$\underline{C}$H-, Ar), 127.6 (>$\underline{C}$H-, *o*-Ar), 127.7 (>$\underline{C}$H-, *p*-Ar), 128.4 (>$\underline{C}$H-, *m*-Ar), 129.6 (>$\underline{C}$H-, Ar), 137.2 (=$\underline{C}$H-), 137.9 (=$\underline{C}$H-, isomer), 129.3 (=Cq), 137.7 (=$\underline{C}$HC(O)-), 154.9 (-OO$\underline{C}$N), 156.8 (OCq, Ar), 169.5 (HN$\underline{C}$=O); ESI-MS: 447.1 $[M+H-Boc+H]^+$, 491.3 $[M+H-56]^+$, 547.3 $[M+H]^+$, 569.2 $[M+Na]^+$.

γ-Methyl ester of N_α-(Carbobenzyloxy)-L-glutamyl-rimantadine

(Table 1, Entry 7 (Z-Glu(OCH_3)-Rim)**:** Yield: 42 %; mp ~ 132-134˚C; UV (C_2H_5OH) λ_{max} = 208 nm; IR (ATR)u_{max}: 3292, 2902, 1730, 1690, 1647, 1514, 1454 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): 0.89 (3H, >CHC$\underline{H}_3$), 1.3-1.7 (12H, 6 x -C$\underline{H}_2$-; rimantadyl-), 1.78 (1H, >CHC$\underline{H}_{2a}$-), 1.89 (4H, 3 x >C$\underline{H}$-, rimantadyl-, + 1H >CHC$\underline{H}_{2b}$-), 2.34 (m, 2H, -C(O)C$\underline{H}_2$-), 3.48 (m, 1H, -HNC$\underline{H}$CH$_2$-), 3.58 (s, 3H, -OC$\underline{H}_3$), 4.04 (m, 1H, -NHC$\underline{H}$CH$_3$), 5.02 (2H, -O-C$\underline{H}_2$-), 7.2-7.5 (5H (Ar) + 2H >N$\underline{H}$); ^{13}C NMR (150 MHz, DMSO-d6): 14.1 (1C, >CH$\underline{C}$H$_3$), 27.7 (-$\underline{C}$H$_2$CH$_2$C(O)-), 27.7 (3C, >$\underline{C}$H-; rimantadyl-), 30.0 (-CH$_2\underline{C}$H$_2$C(O)-), 35.4 (Cq), 36-39 (6C, -$\underline{C}$H$_2$-; rimantadyl-), 51.3 (1C, -O$\underline{C}$H$_3$), 52.0 (-HN-$\underline{C}$HCH$_3$), 54.0 (-C(O)HN-$\underline{C}$H<), 65.4 (-O$\underline{C}$H$_2$-), 127.0-128.5 (5 x =CH-), 155.8 (-CH$_2$O$\underline{C}$(O)-), 170.4 (-HNCHC(O)NH-), 172.7 (-C(O)OCH$_3$). ESI-MS: 457.1 $[M+H]^+$, 479.1 $[M+Na]^+$.

β-Benzyl ester of N-tert-butoxycarbonyl-L-aspartyl-rimantadine

(Table 1, Entry 8 (Boc-Asp(OBzl)-Rim)**:** Yield: 54 %; mp ~ 71-74˚C; UV (C_2H_5OH) λ_{max} =207, nm; IR (ATR)u_{max}: 3331, 2979, 2919, 1729, 1695, 1655, 1514, 1393, 1367 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6): 0.89 (d, 3H, >CHC$\underline{H}_3$), 1.39 (s, 9H, -C(CH$_3$)$_3$), 1.42-1.63 (12H, 6 x -C$\underline{H}_2$-; rimantadyl-), 1.89 (3H, 3 x >CH-; rimantadyl-, 2.89 (m, 2H, >CHC$\underline{H}_2$C(O)O-), 3.46 (m, 1H, -HNC$\underline{H}$CH$_2$-), 4.32 (m, 1H, -NHC$\underline{H}$CH$_3$), 5.12 (s, 2H, -O-C$\underline{H}_2$-), 7.06-7.37 (5H (Ar) +2H >NH); ^{13}C NMR (150 MHz, DMSO-d_6): δ 14.0 (1C, >CH$\underline{C}$H$_3$), 27.7 (3 x -$\underline{C}$H$_3$), (-$\underline{C}$H$_2$C(O)-), 28.1 (3C, >$\underline{C}$H-; rimantadyl-), 36.4-36.6 (6C, -$\underline{C}$H$_2$-; rimantadyl-), 37.7 (-$\underline{C}$H$_2$-), 51.1 (-HN-$\underline{C}$H(CH$_3$)-), 52.2 (-HN-CH<), 65.5 (-O$\underline{C}$H$_2$-), 78.4 (-O$\underline{C}$(CH$_3$)$_3$), 127.7-127.9 (5 x =CH-), 128.3 (=Cq), 136.1 (=$\underline{C}$HC(O)-), 155.3–(C-O$\underline{C}$(O)-NH-), 169.8 (-HNCH$\underline{C}$(O)NH-), 170.1 (-C(O)OCH$_2$-); ESI-MS: 385.1 $[M+H–Boc+H]^+$, 429.0 $[M+H–C(CH_3)_3+H]^+$, 485.1 $[M+H]^+$, 507.3 $[M+Na]^+$.

Antiviral activity assay

Cells and viruses. MDCK cells for the propagation of influenza virus A originated from the collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria, and were grown in a growth medium containing Dulbecco modified

Table 1. Effect of the amino acids linked to rimantadine, amantadine and oseltamivir against the influenza virus A/Aichi/2/68 (H3N2)

Entry	Compound	MTC (μM)	CC_{50}[a] (μM)	IC_{50}	SI (CC_{50}/IC_{50})
1	Fmoc-Tyr(But)-Os	100	306.47±20.92	-	
2	Ac-Cys-Os	100	307.14±11.94	-	
3	Boc-His(Dnp)-Os	56	140.99±20.24	-	
4	Boc-N(CH_3)-Tyr(Bzl)-Os	>1000		-	
5	Boc-N(CH_3)-Tyr(Bzl)-Am	0.3	1.00±0.57	-	
6	Boc-N(CH_3)-Tyr(Bzl)-Rim	100		-	
7	Z-Glu(OCH_3)-Rim	3200		-	
8	Boc-Asp(OBzl)-Rim	32	10.12±1.60	-	
	Rimantadine	100	175	0.2	875
	Amantadine	100	330	1.6	206

CC_{50}: 50% cytotoxic concentration; MTC: maximal tolerance concentration; SI: selective index;
[a] Data are shown as mean ± SD of four independent determinations

Eagles' medium (DMEM) (Gibko BRL, USA), supplemented with 10 % fetal bovine serum, 10 mM HEPES buffer (Merck, Germany) and antibiotics (penicillin 100 IU mL^{-1} and streptomycin 100 μg mL^{-1}). The cells were cultured as confluent monolayers in a humidified atmosphere containing 5 % CO_2 at 37 °C.

Influenza virus A/Aichi/2/68 (H3N2) from the collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, was grown in MDCK cells in a maintenance medium of Dulbecco modified Eagles' medium (DMEM) (Gibko BRL, USA), containing 0.5 % fetal bovine serum, 10 mM HEPES buffer and antibiotics, as well as 3 mg mL^{-1} trypsin (Gibco BRL).

Cytopathic effect (CPE) inhibition test. Monolayer MDCK cells in 96-well microplates (Costar, USA) were inoculated, following the removal of the growth medium, with 0.1 mL virus suspension containing 100 $CCID_{50}$ (cell culture infectious dose 50 %). After 1 h at 37 °C for virus adsorption, the innoculum was washed out and replaced by 0.1 mL of non-cytotoxic 0.5 $\log_{10}$ dilutions in the maintenance medium of the newly synthesized compounds. Each dilution was applied in quadruplicate. Cells that were not inoculated with virus were left for cell controls (with only maintenance medium) and toxicity controls (with respective dilution of the compound in the maintenance medium). Cells inoculated with virus but not treated with a compound were left for virus controls. Then cells were incubated for 48 h in a humidified atmosphere with 5 % CO_2 at 37 °C or until the virus specific cytopathic effect had destroyed 100 % of the cells in the virus control wells. Then cells were stained according to the neutral red uptake procedure and the percentage of CPE inhibition, if present, was calculated using the following formula [15]:

$$\% \text{ CPE} = (OD_{\text{test sample}} - OD_{\text{virus control}})/(OD_{\text{toxicity control}} - OD_{\text{virus control}}) - 100.$$

RESULTS AND DISCUSSION

Chemistry

Despite the extensive efforts have been invested in designing of potential influenza antivirals, the continuing risk of a future pandemic flu remains very real.

Emerging from the restoration of the antiviral activity of amino acid analogues with anti-influenza drugs [12], herein we modify the anti-viral drugs amantadine, rimantadine and oseltamivir with amino acid moiety. The synthetic route for amino acid analogues is outlined in Schemes 1. As shown, the synthesized amides were obtained in low to good yields by the classical EDC/HOBt method of peptide chemistry [13, 14]. The desired compounds (**1-8**) were purified by preparative thin layer chromatography and their structures were assessed by means of melting points, UV, IR, ^{1}H-NMR, ^{13}C-NMR and ESI-MS.

ESI-MS spectra in positive mode of ionization clearly reveal that the monitored base peaks are consistent to anticipated adducts $[M+H]^+$, $[M+Na]^+$ for all target compounds. The formation of amide bond is confirmed in ^{1}H-NMR spectra by presence of a signal for amide proton at δ ~ 6.5-7.5 ppm. Whereas ^{13}C-NMR spectra show a signal at about δ ~175 ppm for carbonyl carbon of amide bond. Additional information is collected from IR spectra bands. The observed absorbance at ~1640-1680 cm^{-1} corresponds to N-C=O group.

Biological activity

According literature data the protection of α-amino- and side chain polar functional groups of amino acids produced the very active anti-influenza compounds [12]. These promising results enforced us to study antiviral activity of protected amino acid analogues with amantadine, rimantadine and oseltamivir.

Preliminary antiviral activities of the synthesized compounds (**1**-**8**) against influenza A (H3N2) were evaluated *in vitro* through their ability to prevent cytopathic effects (CPE) in influenza A virus (H3N2) infected Madin-Darby canine kidney (MDCK) cells. The data of the tested amides (Table 1) were compared to the positive controls-amantadine (Am) and rimantadine (Rim).

The newly synthesized compounds did not reveal an enhanced antiviral activity as compared to the generic antivirals.

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АМИНОКИСЕЛИННИ АМИДИ НА ПРОТИВОГРИПНИ ЛЕКАРСТВЕНИ СРЕДСТВА: СИНТЕЗ И БИОЛОГИЧНО ДЕЙСТВИЕ

Б. Стойкова[1], М. Чочкова [1*], Л. Георгиев[1], Г. Иванова[2], Л. Мукова[3], Н. Николова[3],

Л. Николаева-Гломб[3], Ц. Милкова[1], М. Щиха[4]

[1]Катедра по химия, Югозападен университет „Неофит Рилски“, 2700 Благоевград, България
[2]Департамент по химия и биохимия, Университет в Порто, 4169-007 Порто, Португалия
[3]Институт по микробиология „Стефан Ангелов“, Българска академия на науките, София, България
[4]Департамент по органична химия, Карлов университет, 12843 Прага 2, Чехия

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(Резюме)

Грипните вируси тип А са сред най-вирулентните респираторни патогени, водещи до значителна заболеваемост и смъртност. Високата честота на антигенни вариации на грипния вирус е причина за възникване на пандемични взривове. Ето защо създаването на нови противогрипни средства е изключително атрактивна изследователска област.

В настоящето изследване е разгледан синтеза и е изследвана противогрипната активност на аминокиселинни амиди на римантадин и оселтамивир. Изпитването за антивирусен ефект е проведено *in vitro* спрямо грипен вирус тип А (A/H3N2). Резултатите от скрининга показват, че амидното свързване на аминокиселинните аналози (тирозин, хистидин, аспарагинова и глутаминова киселини) с оселтамивир и римантадин не водят до повишаване противовирусната активност спрямо изпитвания щам.