SYNTHESIS, HYDROLYTIC STABILITY AND ANTIVIRAL STUDIES OF SULFUR-BASED ANTI-INFLUENZA DRUGS

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Received 10 October 2025
Accepted 17 November 2025
DOI: 10.59957/jctm.v61.i1.2026.6

ABSTRACT

Sulfur - containing building blocks represent highly favored scaffolds in various natural or synthetically obtained pharmacologically active molecules. Their diverse functionalities, including thioether, sulfonamide, sulfoxide, and others are often linked with the possibility to inhibit microbial growth and to regulate immune responses. Nowadays, approximately 25 % of all utilized small - molecule pharmaceuticals have been approved as organosulfur drugs or are clinical candidates. Herein, inspired by the medicinal power of such compounds, a series of potential sulfur based ligands against influenza A viruses was obtained by modification of amino group of anti - influenza drugs (rimantadine, amantadine and oseltamivir) and memantine. Furthermore, the structures of novel derivatives bearing versatile sulfur - containing acyl moieties (cysteinyl-, thienyl-) were established by spectroscopic methods (^{1}H NMR, ^{13}C NMR, (ATR)u_{max}, and HRMS). The newly synthesized amides were tested in vitro for antiviral activity against four influenza A viruses (A/Fort Monmouth/1/1947 (H1N1), A/Wuhan/359/1995 (H3N2), A/Jinnan/15/2009 (H1N1), and A/Aichi/2/68 (H3N2). Amongst the evaluated amides 1, 6, and 7 were the most active ones, inhibiting both the A/Fort Monmouth/1/1947 (H1N1) and A/Wuhan/359/1995 (H3N2) influenza virus strains, while amides 3 and 5 showed pronounced antiviral efficacy specifically against the A/Wuhan/359/1995 (H3N2) strain. Moreover, compounds 6 and 7 indicated remarkable inhibitory activity against the oseltamivir-resistant A/Jinnan/15/2009 strain.

Furthermore, the hydrolytic stability of desired amides was monitored in two model pH systems that mimic conditions in the stomach, and blood plasma, including pH 1.0, and 7.4, respectively.

Keywords: sulfur - containing compounds, hydrolytic stability, anti - influenza drugs, influenza A viruses.

INTRODUCTION

Undoubtedly, the viruses are one of the major pathogens responsible for life - threatening diseases in human beings [1]. It has been reported more than 400 pathogenic viral species, recognizing human cells as hosts [2]. Amongst them, stand out those, responsible for influenza, smallpox, acquired immunodeficiency syndrome, hepatitis, rabies, polio, Ebola, and SARS. Indeed, over the last 20 years the respiratory RNA viruses as SARS-CoV, the influenza 2009 H1N1 virus, Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 have been implicated in the recent epidemic and pandemic events [3 - 8].

Even though more than five years have passed since COVID-19 was firstly declared as a pandemic [9], the sense of fear still remains. Such dreadful disaster profoundly affected not only the health, economic, and educational systems [10], but also shook human mobility dynamics [11] worldwide. In particular, COVID-19 and seasonal influenza (flu) are both acute respiratory illnesses, that caused by distinct viruses and have also differences in the clinical characteristics [12]. Indeed, a COVID-19 pandemic resembles the past pandemics provoked by influenza viruses. Given their history and ongoing circulation, influenza viruses remain the most likely drivers of future pandemics [13]. During the pandemic COVID-19 wave, along with the circulation of high prevalence of SARS-CoV-2, the influenza coinfection tremendously increase the patients' mortality rates [14]. Nevertheless, the most individuals very often negligee flu as a common cold [15]. As per the latest WHO' reports, the number of COVID-19 deaths (up to 17 August 2025) are estimated to be >7 100 227 [16], while influenza is liable for 290 000 to 650 000 respiratory deaths annually [17]. For the past 107 years, besides the unexpected scenario in 2019, four influenza pandemics in 1918, 1957, 1968, and 2009 inflicted a significant harm on the human global populations [2, 18].

Unfortunately, the influenza vaccine effectiveness is insufficient to provide 100 % protection in prophylactic against the newly emerging flu strains, arising from antigenic drift. Concerning of the vaccine effectiveness for the last three seasons (2021 to 2024) it has been estimated that one-third of influenza vaccinated individuals were protected [19]. Consequently, the antiviral therapy remains a vital prophylactic and therapeutic approach to combat influenza virus infections.

Currently, several low - molecular weight anti - influenza drugs have been known, comprising the oldest adamantanes class inhibitors - M2 ion channel blockers (amantadine (Am) and rimantadine (Rim)) [20], on the other hand sialic acid analogs - neuraminidase inhibitors (Tamiflu or oseltamivir (Os), zanamivir, and peramivir) [21, 22], and nucleoprotein inhibitor (baloxavir marboxil) [23]. In fact, the defined widespread antiviral resistance of the first class antivirals leads to their clinically avoidance [24]. Therefore, adamantanes have been replaced by neuraminidase inhibitors [25], and baloxavir marboxil [26]. Actually, the established emergence of flu strains with dual resistance to both adamantane and neuraminidase classes of antivirals has caused increasing concern [27]. This enforces enlarged research on the reasons for the development of resistance and ways of overcoming it by functionalization of the existing antiviral drugs. Thus, one possible option to "revive" the antiviral activity of aminoadamantanes is their modification with additional functional groups that could be able to disrupt H^+ transport across the viral membrane. In this regard, following this strategy Shibnev V. A. and co - authors have obtained amino acid derivatives of aminoadamantanes with enhanced inhibitory activity against rimantadine - resistant influenza virus type A (H3N2) and pandemic strain A (H1N1) pdm [28].

Particularly, cysteine is a natural thiol - containing amino acid endows with unique properties arising from the participation of its side chain (-SH group) in different nucleophilic reactions [29]. It can also serve as a precursor for a synthesis of essential molecules, one of which is the endogenous non - enzymatic antioxidant glutathione (gamma - Glutamyl - Cysteinyl - Glycine) [30]. However, being a cysteine analogue, *N*-acetylcysteine (NAC) elicits a significant medicinal impact on human health [31], due to its mucolytic properties [32], anti-inflammatory activity [33], as well as its remarkable antioxidant activity [34]. Various studies have shown for possible advantages of combined treatment of antioxidant NAC with clinically approved anti-influenza drugs, such as oseltamivir [35], and ribavirin [36]. Moreover, another class of *S*-containing compounds with prominent biological activities [28, 37 - 42] are sulfur heterocycles (e. g. thiophen,

dithiolane) that exist in diverse natural compounds and drugs.

Considering the above - mentioned events there is a pressing need to find and design safer broad - spectrum antiviral agents capable to tackle different influenza strains. The current work is focused on synthesis of aminoadamantane or oseltamivir derivatives, incorporating *S* - containing residues (cysteine analogues/ 3-(2-thienyl)acrylic acid moiety or lipoyl moiety), in order to study *in vitro* activities against four influenza A viruses (A/Fort Monmouth/1/1947 (H1N1), A/Wuhan/359/1995 (H3N2), A/Jinnan/15/2009 (H1N1)) and A/Aichi/2/68 (H3N2).

EXPERIMENTAL

Materials and methods

All solvents and reagents used in this study were purchased from Labimex (Sofia, Bulgaria), while the TBTU reagent (*N*-[(1H-benzotriazol-1-yl) (dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide) and 3-(2-thienyl)acrylic acid (TA) were products from BLDpharm. Oseltamivir phosphate (NICPBP, lot number 101096 - 200901), Oseltamivir carboxylate (OsC, Medchem, Princeton, NJ, USA), and amantadine hydrochloride (Am.HCl, Sigma, lot number 665 - 667) were used as positive control drugs in antiviral assays. TLC was performed on precoated Kieselgel 60F_{254} plates (Merck, Darmstadt, Germany), with detection by UV absorption at λ = 254 nm. A spray Ce - PMo reagent, consisting of 10 g $Ce(SO_4)_2$, 25 g $H_3[P(Mo_3O_{10})_4] \times H_2O$, 940 mL H_2O, and 60 mL conc. H_2SO_4, was used for TLC visualization. Flash chromatography of the target amide was performed on a Pure C - 805 system (BÜCHI GmbH) using FlashPure EcoFlex Flash Chromatography Cartridges.

Melting points were determined using capillary tubes on a „Stuart SMP10“ apparatus and were not corrected. UV - spectra were acquired on UV - Vis - NIR spectrophotometer (Agilent Technology 8453) in C_2H_5OH. The Infrared spectra (ATR-IR) were performed on Thermo Nicolet 6700 FT-IR at 4 cm^{-1} resolution and 32 scans over the range 4000 - 650 cm^{-1}. The NMR measurements were performed on a Bruker Ascend neo NMR 600 instrument (Bruker, Billerica, MA, USA) at 600MHz for ^1H and at 151MHz for ^{13}C nuclei, respectively, and on Bruker Avance II + spectrometer (14.09 T magnet), operating at 600.01MHz ^1H frequencies, equipped with 5mm BBO probe with the z - gradient coil. The temperature was maintained at 293 K, using Bruker B-VT 3000 temperature unit with an airflow of 535 L h^{-1}. NMR are reported as chemical shifts (δ) in ppm relative to $(CH_3)_4Si$ as an internal standard. Some of the MS spectra were obtained on a Brucker Esquire 3000 plus instrument, along with HRMS analyses that were performed on a Bruker Compact QTOF - MS (Bruker Daltonics, Bremen, Germany). The data analysis was performed and the mono - isotopic mass values were calculated using Data analysis software v 4.4 (Bruker Daltonics, Germany). The analyses were conducted in the positive ion mode at a scan range from m z^{-1} 50 to 1000, and nitrogen was used as nebulizer gas at a pressure of 4 psi and flow of 3 L min^{-1} for the dry gas.

Method A. General condensation (EDC/ HOBt) method for synthesis of amides (1, 2) of *N*-Boc-Cys(Bzl)-OH with rimantadine hydrochloride or oseltamivir phosphate [43]

1 g (3.2 mmol) of *N*-Boc-Cys(Bzl)-OH, 0.432 g (3.2 mmol) of HOBt (*N*-hydroxybenzotriazole) in 15 ml CH_2Cl_2 was stirred and cooled at 0°C. To the cooled solution was added 0.612 g (3.2 mmol) EDC, and then (after 5 min) 3.2 mmol of the corresponding anti-influenza agent in 5 ml CH_2Cl_2 and 0.35 ml (3.2 mmol) NMM was added. After the reaction was completed (24 h, TLC - control, CH_2Cl_2:CH_3OH = 2.5:0.05; 2.5:0.2) CH_2Cl_2 was evaporated. The residue was dissolved in ethyl acetate and washed sequentially with 5 % $NaHSO_4$, 5 % $NaHCO_3$ and with saturated NaCl to pH = 7. The organic phase was dried over anhydrous Na_2SO_4 and then was evaporated under reduced pressure. The residue was purified by column chromatography (CH_2Cl_2:CH_3OH) with increasing polarity.

Compounds characterization:

Compound 1 (Boc-Cys(Bzl)-Rim, yield 20 %): mp ~ 72 - 74°C; UV (C_2H_5OH) λ_{max} = 207 nm; IR (ATR)u_{max}: 3296, 3063, 3029, 2977, 2901, 2847, 1683, 1644, 1520, 1495, 1452, 1390, 1365, 1246, 1162, 698 cm^{-1}; ^1H-NMR (DMSO-d_6, ppm): d 0.95 (d, 3H, -NHCHC$\underline{H}_3$), 1.45 (s, 9H, -C($C\underline{H}_3$)$_3$), 1.43-1.62 (12H, 6 x >C$\underline{H}_2$), 1.90 (3H, 3 x >C$\underline{H}$-), 2.71 (m, 1H, -C$\underline{H}_{2a}$S-), 3.07 (m, 1H, -C$\underline{H}_{2b}$S-), 3.83 (m, 1H, -NHC$\underline{H}CH_3$), 3.92 (s, 2H, -C$\underline{H}_2$S-), 4.67

(m, 1H, -NHCHC(O)NH-), 5.98 (1H, >NH), 7.28-7.51 (m, 5H, Ar-H), 7.63 (1H, >NH); ESI-MS: 473.3 $[M+H]^+$, 495.3 $[M+Na]^+$, 511.3 $[M+K]^+$.

Compound 2 (Boc-Cys(Bzl)-Os-OEt, yield 48 %): mp ~ 155 - 157°C; UV (C_2H_5OH) λ_{max} = 207 nm; IR (ATR)u_{max}: 3282, 3088, 2973, 2933, 2877, 1714, 1647, 1519, 1495, 1454, 1391, 1366, 1243, 1165, 1055, 700 cm^{-1}; ^{1}H-NMR (DMSO-d_6, ppm): d 0.87 (t, *J*= 7.4 Hz, 3H, -CH_2CH_3), 0.94 (t, *J* = 7.3 Hz, 3H, -CH_2CH_3), 1. 31 (m, 4H, 2 x -CH_2CH_3); 1.49 (m, 3H, -OCH_2CH_3), 1.50 (s, 9H, -$C(CH_3)_3$), 1.83 (s, 3H, -C(O)CH_3), 2.28 (m, 1H, =CCH_{2a}-), 2.58 (m, 1H, =CCH_{2b}-), 2.79 (m, 1H, -CH_{2a}S-), 3.09 (m, 1H, -CH_{2b}S-), 3.51 (m, 1H, >$CHCH_2CH_3$), 3.86 (s, 2H, -CH_2S-), 3.92 (ddd, *J*=10.4, 9.6, 8.7 Hz, 1H, CH_3C(O)NHCH<), 3.97 (m, 1H, -CH_2CHNH-), 4.08 (1H, -OCH<), 4.12 (q, *J*=7.1 Hz, 2H, -CH_2CH_3), 4.79 (m, 1H, -NHCHC(O)NH-), 5.95 (1H, >NH-)6.75 (s, 1H, =CH-), 7.33-7.40 (m, 5H, Ar-H), 7.71 (1H, -C(O)NH-), 7.95 (1H, CH_3C(O)NH-); ESI-MS: 606.3 $[M+H]^+$, 628.4 $[M+Na]^+$, 644.3 $[M+K]^+$.

Compound 3 (H-Cys(Bzl)-Os-OEt, yield 88 %): IR (ATR)u_{max}: 3284, 3085, 2965, 2933, 2877, 1713, 1651, 1538, 1454, 1370, 1243, 1200, 1129, 1055, 699 cm^{-1}; HRMS: *m/z* found: 506.2680 and 528.2498; calculated for $C_{26}H_{40}N_3O_5S^+$ $[M+H]^+$: 506.2683 and for $[M+Na]^+$: 528.2503.

Method B. General experimental way for synthesis of amides (4-11) using TBTU coupling reagent [41]

Compound 4 (TA-Os-OEt, yield 38 %) IR (ATR), NMR and MS were in good agreement with our literature data [40]: mp ~ 234 - 238˚C; UV (C_2H_5OH) λ_{max} = 206, 307 nm

Compound 5 (TA-OsC) [40]

Compound 6 (TA-Rim, yield 54 %) IR (ATR), NMR and MS were in good agreement with our literature data [40]: mp ~ 153 - 157°C; UV (C_2H_5OH) λ_{max} = 206, 305 nm

Compound 7 (TA-Am, yield 62 %) IR (ATR), NMR and MS were in good agreement with our literature data [40]: mp ~ 213 - 216°C; UV (C_2H_5OH) λ_{max} = 206, 302 nm

Compound 8 (TA-Mem, yield 47 %) [40]: mp ~ 176 - 177°C; IR (ATR)u_{max}: 3256, 3073, 2943, 2861, 2839, 1666, 1645,1613, 1558, 1523,1453,1353, 1336,1234, 1212,978, 700, 699 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.63 (s, 1H), 7.57 (d, *J* = 5.1 Hz, 1H), 7.45 (d, *J* = 15.4 Hz, 1H), 7.32 (d, *J* = 3.4 Hz, 1H), 7.09 (dd, *J* = 5.1, 3.6 Hz, 1H), 6.43 (d, *J* = 15.4 Hz, 1H), 2.12 - 2.01 (m, 1H), 1.80 (d, *J* = 2.3 Hz, 2H), 1.62 (q, *J* = 12.0 Hz, 4H), 1.33 (d, *J* = 11.8 Hz, 2H), 1.26 (d, *J* = 12.1 Hz, 2H), 1.12 (s, 2H), 0.82 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 164.3, 140.6, 131.2, 130.7, 128.7, 128.0, 123.1, 52.9, 50.7, 47.5, 42.8, 39.9, 32.3, 30.6, 30.0; HRMS: *m/z* found: 316.172634 and 338.154387; calculated for $C_{19}H_{26}NOS^+$ $[M+H]^+$: 316.172962 and for $[M+Na]^+$: 338.154906.

Compound 9 (LipA-Am, yield 48 %): mp ~ 101 - 103°C; IR (ATR)u_{max}: 3295, 3070, 2904, 2848, 1640, 1540, 1453 cm^{-1}; ^{1}H-NMR (DMSO-d_6, ppm): d 1.33 (m, 2H, >$CHCH_2CH_2CH_2CH_2$-), 1.47 (m, 2H, >$CHCH_2CH_2CH_2CH_2$-), 1.54-1.70 (m, 8H, 3 x –CH_2-; adamantyl- + 1 x >$CHCH_2CH_2CH_2CH_2$-), 1.85 (ddd, *J*=12.8, 6.4, 5.1 Hz, 1H, -CH_{2a}-, 1,2-dithiolane-3-yl), 1.89 (m, 6H, 3 x –CH_2-; adamantyl-), 1.98 (m, 5H, 3 x >CH-; adamantyl- + 1 x >$CHCH_2CH_2CH_2CH_2C(O)$-), 2.40 (ddd, *J*=12.8, 6.2, 5.3 Hz, 1H, -CH_{2b}-, 1,2-dithiolane-3-yl), 3.15 (m, 2H, -S-CH_2-), 3.59 (m, 1H, -S-CH<), 7.19 (1H, >NH); 150 MHz; ^{13}C-NMR (DMSO-d_6, ppm): d 25.1 (-CH_2-), 28.2 (-CH_2-), 28.8 (3 x >CH), 34.1 (-CH_2-), 35.9 (-$CH_2CH_2C(O)NH$-), 36.0 (3 x -CH_2-; adamantyl-), 38.0 (-SCH_2-), 39.8 (-SCHCH_2CH<), 41.0 (3 x -CH_2-; adamantyl-), 50.4 (Cq; adamantyl-), 56.2 (-SCHCH_2-), 171.3 (-C(O)NH); ESI-MS: 340.3 $[M+H]^+$, 362.3 $[M+Na]^+$, 378.3 $[M+K]^+$.

Compound 10 (LipA-Rim, yield 42 %): mp ~ 140 - 144°C; IR (ATR)u_{max}: 3327, 2898, 2845, 1637, 1532, 1449 cm^{-1}; ^{1}H-NMR (DMSO-d_6, ppm): d 1.04 (d, *J*=7.1 Hz, 3H, -CH_3), 1.33-1.56 (12H, 6 x -CH_2-), 1.45-1.64 (m, 6H, 3 x -CH_2-), 1.76 (3H, 3 x >CH-), 2.02 (m, 2H, -CH_2-C(O)-), 2.18 (m, 1H >CH-CH_3), 1.95 (ddd, *J*=12.8, 6.4, 5.1 Hz, 1H, -CH_{2a}-1,2-dithiolane-3-yl), 2.50 (ddd, *J*=12.8, 6.2, 5.3 Hz, 1H, -CH_{2b}-, 1,2-dithiolane-3-yl), 3.21 (m, 2H, -S-CH_2-), 3.28 (m, 1H, -S-CH<), 7.47 (1H, >NH); ESI-MS: 368.4 $[M+H]^+$, 390. 4 $[M+Na]^+$, 406.4 $[M+K]^+$.

Compound 11 (LipA-Os-OEt, yield 37 %): mp ~ 170 - 174°C; UV (C_2H_5OH) λ_{max} = 206 nm; IR (ATR) u_{max}: 3277, 3090, 2964, 2929, 2876, 1714, 1639, 1541, 1461, 1319, 1248, 1053, 732 cm^{-1}; 400 MHz ^{1}H-NMR (DMSO-d_6, ppm): d 0.75 (t, *J*=7.5 Hz, 3H, -CH_2CH_3), 0.82 (t, *J*=7.5 Hz, 3H, -CH_2CH_3), 1.20 (t, *J*=7.0 Hz, 3H, -OCH_2CH_3), 1.25-1.73 (m, 10H, 2 x –CH_2CH_3 + 3 x -CH_2-), 1.76 (s, 3H, -C(O)CH_3), 1.86 (ddd, *J*=12.8, 6.8, 6.1 Hz, 1H, -CH_{2a}-, 1,2-dithiolane-3-yl), 2.01 (dd, *J*= 7.5,

6.8 Hz, -C$\underline{H}_2$C(O)NH-), 2.19 (m, 1H, -C$\underline{H}_{2a}$-), 2.40 (ddd, *J*=12.8, 6.8, 6.1 Hz, 1H, -C$\underline{H}_{2b}$-, 1,2-dithiolane-3-yl), 2.45 (m, 1H, -C$\underline{H}_{2a}$-), 3.14 (m, 2H, -S-C$\underline{H}_2$-), 3.38 (quintet, *J*=5.4 Hz, 1H, -OC$\underline{H}$(CH_2CH_3)$_2$), 3.59 (m, 1H, -S-C$\underline{H}$<), 3.73 (ddd, *J*=10.2, 9.5, 8.6 Hz, 1H, =CC$\underline{H}$CN<), 3.90 (m, 1H, -CH_2C$\underline{H}$(NH)CO), 4.07 (dd, *J*=8.6, 2.4 Hz, 1H, =CHC$\underline{H}$OCH(CH_2CH_3)$_2$), 4.13 (q, *J*=7 Hz, 2H, -OC$\underline{H}_2$$CH_3$), 6.62 (s, 1H, =C$\underline{H}$-), 7.62 (1H, >NH), 7.79 (1H, >NH); ^{13}C-NMR (DMSO-d_6, ppm): d 8.9 (-$\underline{C}H_3$), 9.4 (-$\underline{C}H_3$), 14.0 (-$CH_2\underline{C}H_3$), 22.8 (-(O)CCH_3), 25.1 (2 x -$\underline{C}H_2$-), 25.2 (-$\underline{C}H_2$-), 25.7 (-$\underline{C}H_2$-), 27.9 (3 x -$\underline{C}H_3$), 30.3 (=C$\underline{C}H_2$-), 34.0 (-$\underline{C}H_2$-), 35.5 (-$(CH_2)_3\underline{C}H_2$C(O) NH-), 38.1 (-S$\underline{C}H_2$-), 39.7 (-SCH$\underline{C}H_2$CH<), 47.3 (>N$\underline{C}$HCH_2-), 53.8 (-HN$\underline{C}$HCHCO-), 56.1 (-S$\underline{C}$HCH_2-), 60.4 (-O$\underline{C}H_2CH_3$), 75.1 (>$\underline{C}$H-, -O$\underline{C}$HC=), 81.1 (-OCH(CH_2CH_3)$_2$), 128.4 (=Cq), 138.4 (=$\underline{C}$H-), 165.6 (-C(O)OCH_2CH_3), 169.4 (-C(O)NH-), 171.7 (-$\underline{C}$(O)NH-); ESI-MS: 501.3 $[M+H]^+$, 523.3 $[M+Na]^+$.

Antiviral activity assay

Cells and viruses

Influenza virus A/Fort Monmouth/1/1947 (A/H1N1) from the American Type Culture Collection (ATCC), the oseltamivir-resistant influenza virus A/Jinnan/15/2009 (A/H1N1) and influenza virus A/Wuhan/359/1995(H3N2), generously provided by the Institute for Viral Disease Control and Prevention, China Centers for Disease Control and Prevention, and influenza virus A/Aichi/2/68 (H3N2) from the collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria, were used for the antiviral tests. Viruses were grown in MDCK (Madin-Darby canine kidney) cells from the cell culture collection of the Stephan Angeloff Institute of Microbiology. Cells were cultured at 37°C and 5 % CO_2 in a growth medium containing Dulbecco modified Eagles' medium (DMEM) (Gibko BRL, USA), 10 % fetal bovine serum, 10 mM HEPES buffer (Merck, Germany), and antibiotics (penicillin 100 IU mL^{-1} and streptomycin 100 μg mL^{-1}). For virus propagation and antiviral tests maintenance medium was used, in which serum was reduced to 0.5 % and 3 μg mL^{-1} TPCK-treated trypsin (Worthington, Lakewood, Colorado, USA) was added.

Cellular toxicity

Monolayer MDCK cells in 96-well microplates (Costar, USA) were inoculated with 0.1 mL/well maintenance medium containing serial dilutions of the tested compounds. After 48 h of incubation at 37°C and 5 % CO_2 cells were stained according to the neutral red uptake procedure [44], and the 50 % cytotoxic concentration (CC_{50}) was calculated. The optical density (OD) of each well was read at 540 nm in a microplate reader (Organon Teknika reader 530, Oss, The Netherlands). CC_{50} values were determined by regression analysis.

Cytopathic effect (CPE) inhibition test

Monolayer MDCK cells in 96-well microplates were inoculated with 0.1 mL $well^{-1}$ virus suspension containing 100 dozes 50 % tissue culture infective doses ($TCID_{50}$). Mock-infected wells were left for toxicity and cell controls. After 2 h for virus adsorption cells were washed with phosphate-buffered saline (PBS) and maintenance medium containing serial dilutions of the tested compounds was added. Each dilution was applied in quadruplicate. Cells inoculated with virus but not treated with compound were left for virus controls. Treated cells were further incubated for 48 h at 37°C and 5 % CO_2. For that time virus specific CPE destroyed 100 % of the cells in the virus control wells. Viable cells were stained according to the neutral red uptake procedure and the OD of each well was read at 540 nm in a microplate reader. The percentage of CPE inhibition, if present, was calculated using the Eq. (1) [45]:

$$\% \text{ CPE} = \frac{\left(\text{OD}_{\text{test sample}} - \text{OD}_{\text{virus control}}\right)}{\left(\text{OD}_{\text{toxicity control}} - \text{OD}_{\text{virus control}}\right)} \cdot 100 \qquad (1)$$

where $OD_{test\ sample}$ is the mean value of the ODs of the wells inoculated with virus and treated with the tested compound, $OD_{virus\ control}$ is the mean value of the ODs of the virus control wells, and $OD_{toxicity\ control}$ is the mean value of the ODs of the wells not inoculated with virus but treated with the respective concentration of the test sample. The concentrations that inhibited 50 % of the virus induced CPE, the 50 % inhibitory concentrations (IC_{50}), were determined by regression analysis. The selectivity index (SI) was calculated as the ration between CC_{50} and IC_{50} ($SI = CC_{50}/ IC_{50}$).

Chemical stability measurements by adopted modified procedure [46]

Accurately weighed amounts of the compounds

were dissolved in abs. ethanol to obtain stock solutions with a final volume of 5.0 mL. An aliquot of 0.50 mL of each stock solution was transferred into a 5.0 mL volumetric flask, followed by the addition of 2.0 mL ethanol. The flasks were then filled to the mark with pH 1.0 (HCl) or pH 7.4 (phosphate buffer), yielding working solutions with concentrations in the range of 42 - 56 ppm. The absorbance of the prepared solution with concentration 53.5 ppm was recorded at 203 nm using a UV-Vis-NIR spectrophotometer (Agilent Technology 8453). The measurements performed at 60 - minute intervals over a period of 6 hours.

RESULTS AND DISCUSSION

Chemistry

Since *S*-heterocycles are valuable bio synthons for designing of a variety of structures, the insertion of similar cores in the structure of drugs such as Rim, Am and Mem can significantly affect their physical properties and hence, their pharmacokinetics. Recently, we have revealed that thiophen core emerges as a promising motif for derivatization of M2 and neuraminidase inhibitors for obtaining of potent anti-influenza compounds [40].

In continuation of our research project and inspired by Shibnev et al.'s results for overcoming drug resistance by incorporating of amino acid rest to adamantanes [28, 47], herein a series of *S*-containing hybrids based on molecules as lipoic acid (LipA), thienylacrylic acid or cysteine analogues we used for our ongoing modification of anti-influenza drug.

As illustrated in Scheme 1, following method A, the amide bond formation between cysteine analogue (Boc-Cys(Bzl)-OH) and the corresponding antiviral agent (rimantadine hydrochloride or oseltamivir phosphate) was easily obtained by modified EDC/ HOBt peptide method [43], affording compounds 1 and 2, respectively. Furthermore, the tert-butyloxycarbonyl (Boc-) group in the latter (amide 2) was removed by 50 % TFA/ CH_2Cl_2 and then the treatment of obtained trifluoroacetic acid salt (TFA salts) with NH_4OH afforded free amino function of compound 3 in an excellent yield.

On the other hand, as depicted in Scheme 1 (*method B*), lipoic acid (LipA) was conjugated to the drugs of our choice through TBTU - directed carboxylate activation, providing the desired hybrids 9 - 11 [48, 49].

Moreover, TBTU amidation reagent was applied for the preparation of another group of sulfur hybrids (4 - 8), comprising of 3-(2-thienyl)-acrylic acid (TA) core and adamantanes or Tamiflu moieties (Fig. 1). With the exception of the newly prepared 3-(2-thienyl)acrylic acid amide of memantine (hybrid 8**)**, the rest of compounds (4 - 7) studied herein were obtained previously by means of the modified procedure recently published by us [40].

All synthesized hybrids were isolated in sufficient yields using flash-chromatography purification, and their structures were defined by means of spectral methods (see Experimental section).

Antiviral activity of the tested compounds against influenza viruses *in vitro*

Inspired by our previous anti-influenza studies of thienyl based amides of M2 and neuraminidase inhibitors herein, we compared them with the antiviral activities of the newly obtained *S*-containing derivatives [40]. As shown in Table 1, adamantane compounds 1, 6, and 7 displayed significant inhibitory effects against both the A/Fort Monmouth/1/1947 (H1N1) and A/Wuhan/359/1995 (H3N2) influenza virus strains.

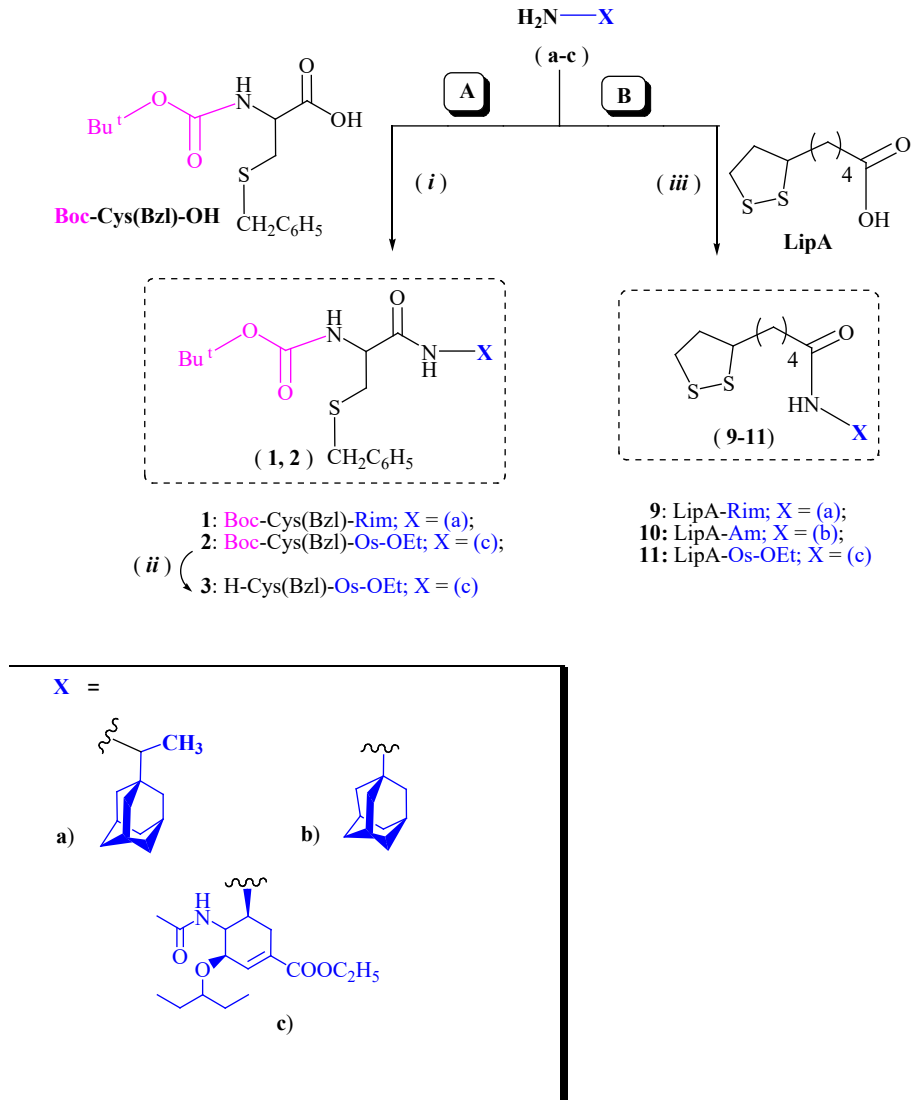
Notably, compounds 6 and 7 further exhibited remarkable inhibitory activity against the oseltamivir-resistant A/Jinnan/15/2009 strain - an observation that underscores their potential to exert broad-spectrum antiviral activity against influenza viruses. Moreover, compounds 3 and 5 showed pronounced antiviral efficacy specifically against the A/Wuhan/359/1995 (H3N2) strain, with selectivity index (SI) values of 58.4 and > 5.2, respectively. Comparing the results of oseltamivir cysteine derivatives (2 and 3) against the A/Wuhan/359/1995 (H3N2) strain, it was shown that amide with free amino group (compound 3) seems to be more active inhibitor than its N-Boc-protected analogue (amide 2).

Additionally, the effect of 3-(2-thienyl)-acrylic acid derivatives (4 - 7) on influenza virus strain A/Aichi/H3N2 was estimated *in vitro* and the obtained results were shown in Table 2.

In this study rimantadine and amantadine were included as positive controls. Unfortunately, the evaluated hybrids 4 - 7 exhibited no activity towards A/Aichi/H3N2.

Chemical stability study of compound 1

Herein, amongst the newly synthesized hybrids,



Scheme 1. Synthesis of the target cysteine and lipoyl derivatives bearing anti-influenza drugs. (i) EDC/HOBt, CH_2Cl_2, t = 0°C for 1 h, then RT (23h); (ii) 50 % TFA/CH_2Cl_2; then NH_4OH; (iii) TBTU, Et_3N, CH_2Cl_2, RT, 4 h.

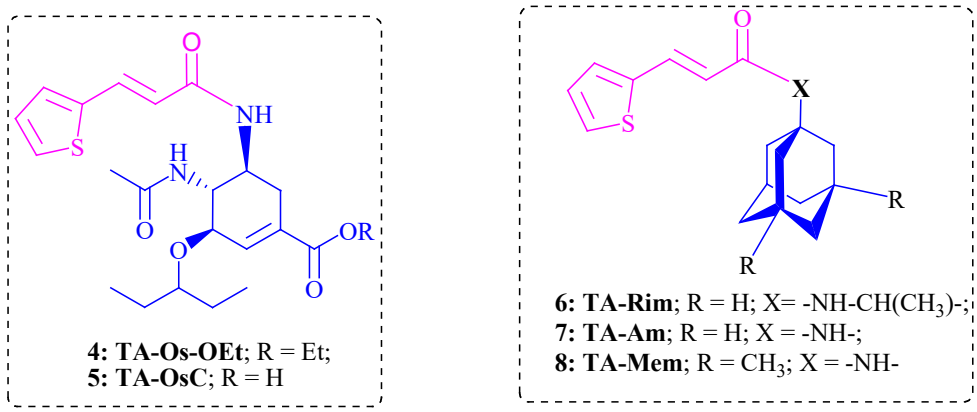


Fig. 1. Structures of synthesized thiophen - containing hybrids.

Table 1. Anti-influenza studies of *S*-containing amides.

Compounds	TC_{50} μg mL^{-1}	A/Fort Monmouth/1/1947		A/Jinnan/ 15/2009		A/Wuhan/ 359/1995	
		IC_{50} μg mL^{-1}	SI	IC_{50} μg mL^{-1}	SI	IC_{50} μg mL^{-1}	SI
1)	96.23	32.08	3.0	> 55.56	-	23.86	4.0
2)	96.23	> 55.56	-	> 55.56	-	> 55.56	-
3)	121.55	NT		NT		2.08	58.4
4)	80.12*	> 18.52*	-*	> 18.52*	-*	> 18.52*	-*
5)	> 210.26*	NT		NT		40.47*	> 5.2*
6)	6.17*	2.06*	3.0*	2.06*	3.0*	2.06*	3.0*
7)	12.84*	3.56*	3.6*	4.79*	2.7*	4.79*	2.7*
Oseltamivir phosphate	577.35*	2.47*	233.7*	115.47*	5.0*	3.12*	185.0*
Oseltamivir carboxylate	14.22	NT		NT		0.034	416.74
Amantadine.HCl	39.22*	0.32*	122.6*	2.86*	13.71*	2.86*	13.71*

*Note: " * " literature data used for comparison [40], " - " means no antiviral activity; NT - not tested; TC_{50}: 50 % toxicity concentration; IC_{50}: 50 % inhibitory concentration; SI: selectivity index (SI = TC_{50}/IC_{50}).*

Table 2. Effect of 3-(2-thienyl)acrylic acid amide on influenza virus strain A/Aichi/H3N2.

Compounds	MTC, μM	CC_{50} [a], mM	IC_{50} [a], mM	SI = TC_{50}/IC_{50}
4) TpA-Os-OEt	> 1000	-	-	-
6) TpA-Rim	1000	-	-	-
7) TpA-Am	> 1000	-	-	-
Rimantadine	100	175	0.2	875
Amantadine	100	330	1.6	206

manifesting significant inhibitory effects against both influenza virus strains (A/Fort Monmouth/1/1947 (H1N1), A/Wuhan/359/1995 (H3N2)), the hydrolytic stability of adamantane compound 1 was selected to be studied.

The chemical stability assessment of amide 1 was provided by monitoring of UV absorption over a period of 6 hours (360 min) at pH 1.0 and pH 7.4 at 37°C (Fig. 2.), which mimic the highly acidic conditions of the gastric environment and blood plasma.

As shown in the graphs (Fig. 2.), the absorption values of hybrid 1 remain nearly constant throughout the entire 6-hour period, with only minor fluctuations within experimental variation. The initial absorption value (~1.5) is maintained under examined conditions, and no significant decrease is observed versus time. Thus, our result indicates that compound 1 is stable under the conditions investigated. As ensuring such preliminary utmost chemical stability represents a highly desirable pharmacokinetic feature in drug development, in order to avoid rapid degradation and to provide sufficient bioavailability. Therefore, the newly synthesized amide (1) could be considered as a promising drug candidate.

CONCLUSIONS

Herein, we have identified a series of hybrids of anti-influenza drugs (rimantadine, amantadine and oseltamivir) and memantine derivativatized by sulfur-

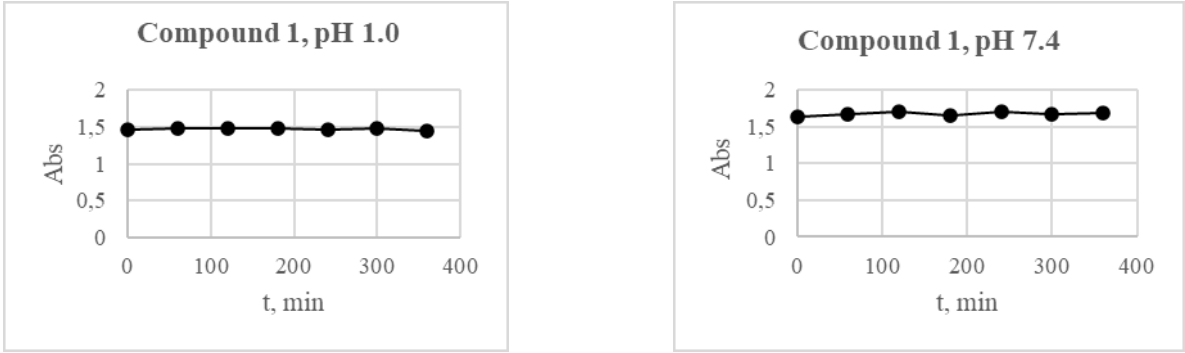


Fig. 2. Chemical stability of amide 1.

containing scaffolds, such as cysteine analogues, 3-(2-thienyl) acrylic acid core and lipoyl- moieties. The performed *in vitro* anti-influenza screening of compounds (1 - 7) indicates the most active compounds inhibiting both the A/Fort Monmouth/1/1947 (H1N1) and A/Wuhan/359/1995 (H3N2) influenza virus strains were adamantane compounds 1, 6, and 7. Moreover, compounds 3 and 5 demonstrated strong antiviral activity against the A/Wuhan/359/1995 (H3N2) strain, with selectivity index (SI) values of 58.4 and 5.2, respectively. In comparison with the positive control - amantadine, the amide 3 displayed higher activity. Thus, 3 and 5 can be potential lead structures for the development of future antiviral agents.

Acknowledgements

This research is supported by the Bulgarian National Science Fund project № KP-06-H79/7 (15.12.2023).

Authors' contributions

M.Ch.: conceptualization, experimental investigation, and writing of the original version; B.St., P.P., H.Y., N.P., Y.Li, M.Št., L.N.-G., J.Sv.: experimental investigation.

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