A synthetic method for the promising new drug oseltamivir ethoxysuccinate is described in detail. Various conditions for obtaining the target substance are considered. Its complete physicochemical characteristics are given. The obtained agent is shown to be effective against influenza virus A (H1N1)pdm09.

Keywords: oseltamivir salts, antiviral activity.

An epidemic of influenza virus is currently a challenging problem for mankind. Oseltamivir, zanamivir, peramivir, and laninamivir are long-acting neuraminidase inhibitors designed to treat and prevent influenza virus [1]. The mechanism of action of these drugs is based on blockage of the enzyme neuraminidase. Oseltamivir, which is perorally active, is most interesting although it is toxic and has several side effects [2]. Oseltamivir itself and its decomposition products in the gastrointestinal tract (GIT) are responsible for the toxicity [3]. It was assumed that organic salts of oseltamivir could be used to accelerate its absorption in the GIT and thereby reduce its decomposition and, correspondingly, its toxicity.

Therefore, research on the replacement of the phosphate salt by a less toxic salt has become popular. Salts that would be just as potent antivirals, have good water-solubility, and crystallize readily and directly from the reaction mixture have been examined.

The possibility of using several pharmaceutically acceptable oseltamivir salts such as the hydrochloride, hydrobromide, nitrate, sulfate, citrate, formate, fumarate, maleate, acetate, p-toluenesulfonate, benzenesulfonate, and glucuronide has been reported [4, 5]. However, the chemical and physical properties of the above compounds and their biological activities were not given. A method for purifying oseltamivir base via formation of its tartrate was described in detail. However, the pharmacological properties of this compound were not disclosed [6].

Oseltamivir phosphate forms upon treatment of oseltamivir base with an equivalent amount of ortho-phosphoric acid in EtOAc. The salt forms in 4–5 h at reaction temperatures ≤45°C. Then, the product crystallizes in 84% yield and 98.9% purity in another 4–5 h upon slow cooling to room temperature. This process is rather lengthy from a technological viewpoint. Therefore, research was aimed at discovering oseltamivir salts that would not only simplify the formation and isolation of the salt but also preserve the pharmacological properties at the level of oseltamivir phosphate.

The stability of the products is one condition for using oseltamivir salts as pharmaceutical substances. Previously, the oxalate, malonate, maleate, succinate, malate, fumarate, citrate, and ethoxysuccinate salts of oseltamivir were investigated. Their physicochemical stability and preservation of the crystal form and color were evaluated [7]. Oseltamivir ethoxysuccinate was selected for further research based on the obtained results and the biological activity of the compounds that were calculated using the PASS Refinder program [8].

An original method for preparing ethoxysuccinic acid (IV) from maleic anhydride (I) was developed [9] (Fig. 1).

The reaction of I with aluminum ethoxide [Al(OCH₃)₂] under these conditions formed intermediate aluminate ester II, which was converted into IV upon treatment first with base and then with acid. The target acid was obtained according to this method in 96% yield and 99% purity according to
HPLC. This made the proposed method significantly more efficient than the method using the reaction of diethyl maleate with sodium ethoxide (80% yield) [10]. Oseltamivir salt VI was readily obtained in EtOAc or Me₂CO at room temperature (Fig. 2). The product precipitated as white crystals from the reaction mixture in 2–4 h at room temperature and did not require recrystallization. The yield of the crystalline product was 96% with 99.8% purity according to HPLC analysis.

The structure of product VI was fully confirmed by physicochemical methods. Its pharmacological potential was demonstrated in comparative tests with Tamiflu®. Also, studies of the acute toxicity in the dose range from 500 to 2000 mg/kg could not determine the LD₅₀ for oseltamivir ethoxysuccinate because large volumes of doses could not be administered and animals in experimental groups did not die. The LD₅₀ lay beyond the limits of the studied doses.

**EXPERIMENTAL CHEMICAL PART**

Melting points were determined on a Stuart Model SMP 30 apparatus (England). IR spectra were recorded on an Infralum FT-801 instrument (Russia). PMR and ¹³C NMR spectra were recorded on a Bruker AM-400 instrument (Germany) at operating frequency 400.13 MHz for ¹H and 100.61 MHz for ¹³C. Chromatographic analysis used an Agilent 1200 HPLC with an MS detector (USA). Elemental analyses were performed on a Thermo Finnigan Flash EA 1112 CHNSO elemental analyzer (Italy).

Oseltamivir base was obtained by the literature method [11]. It was crystallized by adding the liquid residue remaining after concentration to the calculated amount of Et₂O (600 mL of Et₂O per 100 g of liquid residue).

**Preparation of ethoxysuccinic acid (IV)**

A 700-mL stainless-steel autoclave was loaded with maleic anhydride (127.5 g), aluminum ethoxide (84.3 g), anhydrous EtOH (300 mL), and a magnetic stirrer and sealed. The autoclave was placed into a silicone bath. The stirring and heating were started. The temperature of the silicone bath was raised after 40–60 min to 132–136°C. The pressure rose to 4.6 atm. The reaction mixture was held at a pressure >4.3 atm for 4 h and then cooled to 45–50°C. The autoclave was opened.

The reaction mixture was stirred, treated with a solution of NaOH (105.6 g) in distilled H₂O (1050 mL), refluxed, and concentrated to a volume of 900–1000 mL over 2.5 h by distilling the EtOH. The residue was cooled and adjusted to pH 6–7 using conc. HCl (37%) to afford a suspension that was stirred for 30 min and filtered to remove Al(OH)₃.

The filtrate was treated with conc. HCl (222 mL), held at room temperature for 1 h, and evaporated to constant weight.

The obtained residue was treated with dichloroethane (2100 mL), refluxed for 1.5 h, and filtered. The filtrate was stirred for 14 h. The resulting precipitate was filtered off and dried in air to afford ethoxysuccinic acid (199.4 g), mp 86–88°C. PMR spectrum (Me₂CO-d₆, δ ppm): 2.04–2.08 (m), 2.60–2.84 (m), 3.48–3.59 (m), 3.58–3.78 (m), 4.24–4.30 (m), 8.0–11.0 (br.s). ¹³C NMR spectrum (Me₂CO-d₆, δ ppm): 14.54; 37.31; 65.99; 74.93; 170.86; 172.18. Empirical formula: C₆H₁₀O₅. Calc., %: C 44.45; H 6.22; O 49.34. Found, %: C 44.53; H 6.18; O 49.29.

**Preparation of oseltamivir ethoxysuccinate (VI)**

Ethoxysuccinic acid (9.1 g, 55.8 mmol) was dissolved in EtOAc (150 mL). The resulting solution was treated with a solution of oseltamivir base (15.8 g, 50.75 mmol) in EtOAc (100 mL) and held at 45°C. The warm solution was filtered.
through a Schott glass filter under vacuum. The filtrate was stirred for 16 h. The resulting precipitate was filtered off and dried in air to afford ethyl (3S,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)cyclohex-1-ene-1-carboxylate ethoxysuccinate as white crystals with mp 108 – 110°C. PMR spectrum (DMSO-d6, /c100 ppm): 0.77 – 0.85 (m); 1.04 – 1.09 (m); 1.20 – 1.25 (m); 1.34 – 1.43 (m); 1.87 (s); 2.26 – 2.39 (m); 2.50 – 2.57 (m); 2.77 (dd); 3.20 – 3.27 (m); 3.31 – 3.39 (m); 3.57 – 3.63 (m); 3.71 – 3.78 (m); 4.13 – 4.18 (m); 6.65 (s); 8.11 (d). 13C NMR spectrum (DMSO-d6, /c100 ppm): 9.33; 9.83; 14.51; 15.67; 23.65; 25.53; 26.08; 29.19 (CH2); 29.22; 49.35; 53.14; 64.87; 74.65; 76.21; 81.65; 127.65; 138.81; 165.58; 172.95; 174.92. Empirical formula: C16H28N2O4·C6H10O5. Calc., %: C 55.64; H 8.01; O 5.90. Found, %: C 55.64; H 7.93; N 6.01.

EXPERIMENTAL BIOLOGICAL PART

The therapeutic effect of VI was comparatively assessed using Tamiflu® as a reference drug to treat BALB/c inbred mice infected with influenza induced by an adapted variant of the pandemic influenza A virus (H1N1)pdm09. Virus containing MA1-BALB/c strain material was produced. LD50 values were determined to calculate the working dose for infecting the experimental mice. The doses of the administered drugs for the experimental animals were determined.

EXPERIMENTAL ANIMAL GROUPS

The experiments used 140 female BALB/c mice aged 6 – 8 weeks (18 – 24 g). The mice were kept under standard vivarium conditions at 18 – 26°C with a 12:12-h (day-night) lighting regime in plastic cages (Velaz). The mice were divided into six groups. Group 1 (30 animals) received intragastrically oseltamivir ethoxysuccinate (10 mg/kg/d) in distilled H2O (200 L). Group 2 (30 animals) received intragastrically oseltamivir ethoxysuccinate (20 mg/kg/d) in distilled H2O (200 L). Group 3 (30 animals) received intragastrically Tamiflu® (10 mg/kg/d) in distilled H2O (200 L). Group 4 (30 animals) received intragastrically Tamiflu® (20 mg/kg/d) in distilled H2O (200 L). Group 5 (10 animals) acted as a negative control and did not receive a drug. Group 6 (10 animals) acted as a positive control, was not infected, and received intragastrically distilled H2O (200 L). The drugs and solvent (distilled H2O) were administered to the mice 5 d after infection.

EXPERIMENTAL MODEL

Mice of groups 1 – 5 were infected intranasally with an adapted variant of the pandemic influenza A virus (H1N1)pdm09 deposited in GenBank under the name A/Tomsk/273-MA1/2010(H1N1pdm09) (MA1-BALB/c) (KM277585-KM277592) at a dose of 10 LD50 in phosphate buffered saline (PBS, 50 μL). Mice of group 6 were administered intranasally PBS (50 μL). Mice of groups 1 – 4 were administered the drugs after 2 h; of group 6, distilled H2O. The animals were observed for 14 d, during which their behavior and appearance were assessed, body mass was measured, body temperature in an ear canal was measured in °C using a Thermoval duo scan infrared electronic thermometer (Hartmann), the number of deceased mice was counted, and the average lifespan (ALS) was estimated.

### TABLE 1. Comparative Analysis of Experimental Anti-influenza Activities of VI and Tamiflu® in BALB/c Mice Infected with an Adapted Variant of Pandemic Influenza A Virus (H1N1)pdm09

| Parameters                                                   | Experimental BALB/c mouse groups |
|--------------------------------------------------------------|---------------------------------|
| Group 1, VI, 10 mg/kg/d                                      | 19.5 ± 0.2 g                    |
| Appearance of first signs of disease as statistically significant body-mass loss (after infection), d | 3                                |
| Lowest threshold of body-mass loss in animals during experiment, d | 8                                |
| Maximum body-mass loss during experiment, %                  | 29.4                            |
| Lethal outcomes recorded, d                                  | 9, 10, 11                       |
| Total number of deceased animals                             | 4                               |
| Lethality, %                                                 | 13.3                            |
| Survival, %                                                  | 86.7                            |
| ALS, d                                                       | 13.33 ± 0.32                    |

Note: ALS, average lifespan; # statistically significant difference between groups VI (20 mg/kg/d) and Tamiflu® (20 mg/kg/d) (χ2-criterion); * Mann–Whitney criterion.
RESULTS AND DISCUSSION

Table 1 compares the characteristics of the therapeutic effects of VI and Tamiflu® (oseltamivir phosphate) for treating influenza infection of BALB/c laboratory mice induced by an adapted variant of pandemic influenza A virus (H1N1)pdm09.

An analysis of the series of experimental results found that:

the first signs of the disease in the animals as a statistically significant body-mass loss was recorded uniformly on the third day in all four experimental groups;

the greatest body-mass loss of the animals during the experiment was observed for group 1 that received VI (10 mg/kg/d); the lowest body-mass loss, group 2 (VI, 20 mg/kg/d) and group 3 that received Tamiflu® (10 mg/kg/d);

the largest number of lethal outcomes among the experimental animals was recorded for group 4 that received Tamiflu® (20 mg/kg/d) and was 6 of 30 infected animals; the smallest number, for group 2 that received VI (20 mg/kg/d) and was 1 of 30 infected animals;

the longest ALS among the four mouse groups infected with 10 LD₅₀ by strain MA1-BALB/c was noted for group 2 that received VI (20 mg/kg/d) and was 13.97 ± 0.03 d; the shortest ALS among infected mice, group 3 that received Tamiflu® (10 mg/kg/d) and was 12.97 ± 0.42 d. The ALS of the negative control group that did not receive the anti-influenza drugs was 8.30 ± 0.62 d;

the lowest percent survival was noted for group 4 (Tamiflu®, 20 mg/kg/d) and was 80%; the highest percent survival, group 2 (VI, 20 mg/kg/d) and was 96.7%.

Thus, the antiviral effect of oseltamivir ethoxysuccinate was found to be comparable to that of widely used oseltamivir phosphate. The new drug exhibited lower toxicity. The results allowed the new drug to be considered a promising compound for further studies and for designing a new antiviral agent based on it.

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