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CHARACTERISATION AND STUDY OF 1- [2- (2-BENZOYLPHENOXY) ETHYL] -6-METHYLURACIL MECHANISM OF ACTION

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The aim of the study is to identify 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil using various methods of analysis, as well as to study its action mechanism against wild-type and mutant forms of HIV-1 reverse transcriptase (RT).

Materials and methods. To characterize the structure of the test substance, a few kinds of analysis (X-ray diffraction, elemental, thermal) as well as a few kinds of spectroscopy (UV, IR, and NMR) have been used. The study of the action mechanism of the compound as a potential drug was carried out by evaluating the inhibitory activity against HIV-1 RT wild-type and its mutant forms corresponding to drug-resistant viral strains.

Results. The studies have been carried out to confirm the structure of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil. The UV spectrum has a pronounced absorption maximum when measuring a solution of the substance in tetrahydrofuran at the concentration of 0.10 mg/ml. In the IR spectrum, there are specific bands in the range of 4000-370 cm^{-1}. These factors make it possible to use UV and IR spectra to identify the test compound in the substance. It has also been established that the number and mutual arrangement of functional groups, the integrated intensity of signals in the 1H-NMR spectrum, as well as the structure of the carbon skeleton, correspond to the structure of 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil. The results of studying the action mechanism showed that the test compound is an effective inhibitor of wild-type HIV-1 RT with an inhibition constant of 0.2 μM, as well as an enzyme inhibitor (mutation G190A) with an inhibition constant of 8 μM; enzyme (mutation Y181C) with an inhibition constant of 10 μM, as well as a reverse transcriptase (RT) inhibitor (mutation L100I, K103N, V106A) and a double mutant K103N / Y181C with an inhibition constant of more than 20 μM.

Conclusion. As a result of the performed X-ray structural, elemental, ^{1}H-NMR and ^{13}C-NMR analyzes, the structure of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil has been confirmed. The possibility of using UV, IR and NMR spectroscopy, as well as thermal analyzes to confirm the authenticity during the verification of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil, has been shown. The developed methods can be used in the quality control and included in the draft of practice guidelines for the investigated substance. The studies of the action mechanism of the compound of HIV-1 RT reverse transcriptase have shown that this compound belongs to the group of non-nucleoside reverse transcriptase inhibitors (NNRTIs) of HIV-1.

Keywords: 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil; identification; X-ray structural analysis; thermal analysis, elemental analysis; UV spectroscopy; IR spectroscopy; NMR spectroscopy; action mechanism; HIV-1 reverse transcriptase

Abbreviations: TGA – thermal gravimetric analysis; DSC – differential scanning calorimetry; IR spectroscopy – infrared spectroscopy; NMR spectroscopy – nuclear magnetic resonance spectroscopy; HIV – human immunodeficiency virus; RT – reverse transcriptase; NNRTIs – non-nucleoside reverse transcriptase inhibitors

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ХАРАКТЕРИЗАЦИЯ И ИССЛЕДОВАНИЕ МЕХАНИЗМА ДЕЙСТВИЯ 1-[2-(2-БЕНЗОИЛФЕНОКСИ) ЭТИЛ]-6-МЕТИЛУРАЦИЛА

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Цель – идентификация 1-[2-(2-бензоилфенокси)этил]-6-метилурацила с использованием различных методов анализа, а также исследование его механизма действия в отношении дикого типа и мутантных форм обратной транскриптазы (ОТ) ВИЧ-1.

Материалы и методы. Для характеризации структуры исследуемого вещества использовали рентгеноструктурный анализ, элементный анализ, термический анализ, а также УФ-, ИК- и ЯМР- спектроскопии. Изучение механизма действия соединения, как потенциального лекарственного средства, проводили путем оценки ингибирующей активности в отношении ОТ ВИЧ-1 дикого типа и ее мутантных форм, соответствующих лекарственно-устойчивым штаммам вируса.

Результаты. Проведены исследования, подтверждающие структуру 1-[2-(2-бензоилфенокси)этил]-6-метилурацила. УФ-спектр имеет выраженный максимум поглощения при измерении раствора субстанции в тетрагидрофуране в концентрации 0,10 мг/мл, в ИК спектре наблюдаются специфичные полосы в области 4000–370 $см^{-1}$, что позволяет использовать УФ и ИК спектры для идентификации исследуемого вещества в субстанции. Также было установлено, что количество и взаимное расположение функциональных групп, интегральная интенсивность сигналов в спектре ^{1}H-ЯМР, а также строение углеродного скелет, соответствуют структуре 1-[2-(2-бензоилфенокси)этил]-6-метилурацила. Результаты изучения механизма действия показали, что исследуемое соединение является эффективным ингибитором ОТ ВИЧ-1 дикого типа с константой ингибирования 0,2 μM, а также ингибитором фермента (мутация G190A) с константой ингибирования 8 μM; фермента (мутация Y181C) с константой ингибирования 10 μM, а также ингибитором ОТ (мутация L100I, K103N, V106A) и двойном мутанте K103N/Y181C с константой ингибирования более 20 μM.

Заключение. В результате проведенных рентгеноструктурного, элементного, ^{1}H-ЯМР и ^{13}C-ЯМР анализов была подтверждена структура 1-[2-(2-бензоилфенокси)этил]-6-метилурацила. Показана возможность применения УФ-, ИК- и ЯМР-спектроскопии, а также термических анализов для подтверждения подлинности при входном контроле качества 1-[2-(2-бензоилфенокси)этил]-6-метилурацила. Разработанные методы могут быть использованы в контроле качества и включены в проект НД на исследуемую субстанцию. Исследования механизма действия соединения в отношении ОТ ВИЧ-1 показали, что данное соединение относится к группе ненуклеозидных ингибиторов обратной транскриптазы (ННИОТ) ВИЧ-1.

Ключевые слова: 1-[2-(2-бензоилфенокси)этил]-6-метилурацил; идентификация; рентгеноструктурный анализ; термический анализ, элементный анализ; УФ-спектроскопия; ИК-спектроскопия; ЯМР-спектроскопия; механизм действия; обратная транскриптаза ВИЧ-1

Сокращения: ТГА – термогравиметрический анализ; ДСК – дифференциальная сканирующая калориметрия; ИК-спектроскопия – инфракрасная спектроскопия; ЯМР-спектроскопия – спектроскопия ядерного магнитного резонанса; ВИЧ – вирус иммунодефицита человека; ОТ – обратная транскриптаза; ННИОТ – ненуклеозидный ингибитор обратной транскриптазы

INTRODUCTION

The HIV pandemia is the most urgent problem and still an open challenge in the world public health. Despite the fact that, according to Rospotrebnadzor, it was possible to reduce the growth rate of new HIV infections from 13.4% in 2012 to 0.9% in 2017, the epidemiological situation remains severe.[1] Thus, according to the preliminary data, in 2019, 94,668 new cases of HIV infec-

[1] Resistance to HIV and other socially dangerous diseases: some indicators for 6 years, Government of the Russian Federation, 11.04.2018, Available from: http://government.ru/info/32200/. Russian

tion[2] were detected in the Russian Federation, and the number of people living with HIV in the world, reached approximately 38.0 million.[3]

Nevertheless, the HIV infection continues to be an incurable disease. Its danger is explained by the unique effect of the virus on the human body: the reproduction of the virus in the cells of the immune system does not only make the virus less vulnerable to the action of the latter, but also contributes to the development of other infectious diseases [1]. Consequentially, bacterial and viral diseases caused by opportunistic infections – pneumonia, herpesvirus, as well as cancer, damage to the cardiovascular, gastrointestinal and nervous systems – are often developed in HIV-infected people. It is these phenomena that are the main causes of death in the HIV-infected [2]. A modern approach to antiretroviral therapy is aimed at prolonging and improving the quality of patients' lives [3]. Currently, the best method of treating the HIV infection is highly active antiretroviral therapy (HAART), which involves the use of several active substances with different mechanisms at the same time. These are: at least one drug from the group of HIV nucleoside reverse transcriptase inhibitors (NRTIs) in combination with a HIV non-nucleoside reverse transcriptase inhibitor (NNRTI) and / or inhibitors of other classes [4]. HAART drugs are constantly improving, and the development of new pharmacological units is gaining strength due to the variety of side effects and toxicity, as well as the development of drug resistance in strains [5, 6].

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are a promising group of antiretroviral drugs, which are organic compounds of various classes with a significant proportion of aromatic hydrophobic radicals [7]. These are noncompetitive enzyme inhibitors interacting with the allosteric center of reverse transcriptase, affecting the mobility and flexibility of the polymerization center, which ultimately leads to a decrease in the enzyme efficiency [8-10]. The inhibitory effect of drugs manifests itself in several ways, for example, the binding of nevirapine causes the translation of the hydrophobic residues position, and, as a result, the tertiary structure of the reverse transcriptase protein expands [11]. It also manifests itself due to the influence on the dynamic processes of RT with a nucleic acid matrix [12–14]. There are two generations of NNRTI drugs. The first class includes nevirapine, efavirenz and delaverdine, and the second one includes etravirine and rilpivirine [15, 16]. However, despite a slow development of resistance to the second generation NNRTIs, the HIV mutant strains allowing the virus to resist the action of these drugs, are already encountered in practice [17].

Currently, the approaches to the development of new NNRTIs include the following factors: increasing positional adaptability and conformational flexibility in the drug binding pocket [18]; targeting conserved residues in the binding pocket [19, 20]; improving physicochemical properties with the help of prodrugs, or introducing solubilizing groups [21].

As a result of the multi-year research carried out at the Department of Pharmaceutical and Toxicological Chemistry, as well as at the Research Institute of Pharmacology of Volgograd State Medical University (the Ministry of Health of Russia) in collaboration with scientists from the Institute of Molecular Biology n. a. V.A. Engelhardt (Federal Agency for Scientific Organizations), virologists from the USA and Western Europe (a pharmaceutical company of ImQuestBioSciences Inc., USA; Rega Institute for Medical Research, Belgium), a new class of highly active non-nucleoside inhibitors of viral reproduction has been discovered [22, 23].

Among other compounds, 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil (Fig. 1) showed a high ability to suppress the reproduction of HIV-1 *in vitro.* The compound also suppressed the reproduction of mutant HIV-1 strains and had a resistance profile close to that of efavirenz [24, 25].

The obtained results of preclinical studies make it possible to consider the proposed compound as a promising drug candidate for the treatment of HIV-1 infection.

In the course of the pharmacy development, high standards are being imposed on the quality and safety of medicines. In this regard, it becomes necessary to use research methods in the pharmaceutical analysis that allow achieving maximum specificity and reliability of the results [26]. Thus, the requirements for the use of UV, IR, NMR spectroscopy and a thermal analysis, are increasingly being introduced into the drafts of regulatory documents for pharmaceutical substances.

THE AIM of the study is to identify 1-[2- (2-benzoylphenoxy) ethyl] -6-methyluracil using X-ray, thermal and elemental analyzes, UV, IR, NMR spectroscopy, as well as to study its action mechanism against wild-type and mutant forms of HIV-1 reverse transcriptase (RT).

MATERIALS AND METHODS

The objects of the study were the samples of the pharmaceutical substance 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil, provided by Volgograd State Medical University of the Ministry of Health of Russia. The studied substance is a crystalline powder, practically insoluble in water and organic solvents.

[2] Help HIV infection in the Russian Federation in 2019 (prepared at the Federal Scientific and Methodological Center for the Prevention and Control of AIDS, Central Research Institute of Epidemiology, Rospotrebnadzor). Available from: http: //www.hivrussia.info/wp-content/uploads/2020/02/VICH-infektsiya-v-Rossijskoj-Federatsii-na-31.12.2019.pdf. Russian

[3] WHO HIV / AIDS Fact Sheet, Available from: https: //www.who.int/ru/news-room/fact-sheets/detail/hiv-aids. Russian

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X-ray diffraction study

An X-ray diffraction study of the compound was carried out on a Bruker APEX II diffractometer (Bruker, Germany). The structures were solved by the direct method and refined by the geometric least squares mean (LSM) in the anisotropic full-matrix approximation in terms of the structure factor (F^2_{hkl}). Hydrogen atoms were calculated geometrically and refined with restrictions imposed on the C-H bond lengths and their isotropic displacement parameters. All calculations were performed using ShelXl, SHELXT, and Olex-2 programs.[4,5,6]

X-ray phase study

An X-ray phase study was performed to identify possible polymorphs. The composition investigations of the sample of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil by a powder X-ray diffraction, were performed on a Bruker D8 Advance diffractometer (Bruker, Germany) equipped with a nickel β-filter and a system of controlled slits for monochromatization (l[CuKα] = 1.5418 Å), and a position-sensitive detector LynxEye, in the angular range of 4-60° with a step of 0.02° anglewise**.** A certain amount of the substance was ground in a mortar and applied to a flint plate as suspension in heptane, and then the resulting sample was dried. After obtaining the diffraction data on the results of a single crystal study, the theoretical diffraction pattern was calculated and compared with the experiment. The dependence of the background on the 2q angle was modeled using a series of Chebyshev polynomials up to the fifth order. To take into account the features of the device, the method of fundamental parameters determined in advance using a sample of lanthanum boride LaB6, was used. All calculations were performed using the TOPAS program[7].

Thermogravimetric analysis and differential scanning calorimetry

To determine the thermal properties of the substance, the thermal analysis methods were used – a thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). The TGA of the test compound was carried out on a Derivatograph-C device (MOM, Hungary) at the heating rate of 10° C / min. The data obtained were graphically recorded in the form of curves: thermogravimetric (TG), differential thermogravimetric (DTG), and differential thermal (DTA). On the derivatogram, the TG curve shows the change in the sample mass during the study period, and the DTG curve shows the decomposition rate and is useful for accurately assessing the decomposition steps. The DTA reflects the differentiation of thermal effects, contains information on endo- and exothermic maxima, and is used for a qualitative assessment of the derivatogram. The experimental TGA data were processed using the Winder C program. The DSC studies were performed on a DSC-822e device (Mettler-Toledo, Switzerland) in the temperature range from –145 to +260°C, at the heating rate of 10°C/min. All calculations were performed using the STAR^e program.

Elemental analysis

Elemental analysis was performed on an automatic CHN analyzer VarioMicrocube (Elementar, Germany). Acetanilide (71.098% C; 6.71% H, 10.36% N) was used as a standard sample. The carrier gas was helium, the oxidizing agent was high purity oxygen. The oxidizing column was filled with copper oxide, and the reducing column was filled with wire copper. The combustion of standard weighed samples and 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil substance, pre-selected in tin capsules and weighed on an XP6 ultramicrobalance (MettlerToledo, Switzerland) with an accuracy of 0.001 mg, was carried out at the temperature of 950° C. The reduction of the combustion products on the wire copper was carried out at 550° C. The gaseous destruction products were separated on a chromatographic column and detected in a katharometer. The calculation of the determination results was carried out automatically according to the program supplied with the device.

Ultraviolet and visible Spectrophotometry

In the UV-visible range, the spectra were recorded on a Cary 4000 spectrometer (Varian, USA) by measuring the absorption of radiation in a cuvette with a substance solution at the concentration of 0.1 mg / ml. The sample was dissolved in a volumetric flask in tetrahydrofuran (THF, spectroscopic grades, “Component-reagent”), as well as in dimethyl sulfoxide (DMSO, UV-IR-HPLC-GPC grades, Panreac). The measurements of the obtained solutions were carried out in a quartz cuvette with an optical path length of 1.00 mm (Hellma). The data were processed in the software of the WinUV spectrometer (Varian).

Infrared spectrometry

The experimental work was carried out on a Vertex 70 FT-IR spectrometer (BrukerOptik GmbH, Germany). The samples for recording the spectra were prepared by direct pressing with optically pure potassium bromide; the spectra were measured in the systematic scanning mode in the range of 4000–370 cm^{-1}. A diffuse reflection attachment (Shimadzu, Japan) was used to record the absorption spectrum in the near IR area. All data were processed using the OPUS spectrometer control software (Bruker, Germany).

[4] Dolomanov O.V., Bourhis L.J., Gildea R.J., Howard J.A.K., PuschmannH. OLEX2: a complete structure solution, refinement and analysis program. J. Appl. Cryst. 2009;42: 339–341. DOI: 10.1107/S002188980804272

[5] Sheldrick G.M. SHELXT – Integrated space-group and crystal-structure determination. Acta Cryst. 2015; A71: 3–8. DOI: 10.1107/S2053273314026370

[6] Sheldrick G.M. Crystal structure refinement with SHELXL // ActaCryst. – 2015. – V. C71. – P. 3–8. DOI: 10.1107/S2053229614024218.

[7] Bruker AXS: TOPAS V4: General profile and structure analysis software for powder diffraction data. – User’s Manual, Bruker AXS, Karlsruhe, Germany, 2009: 72 p.

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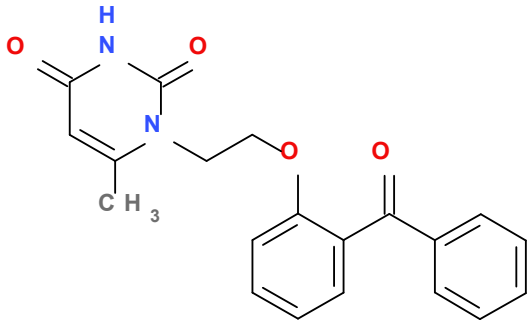

Figure 1 – Structural formula of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil

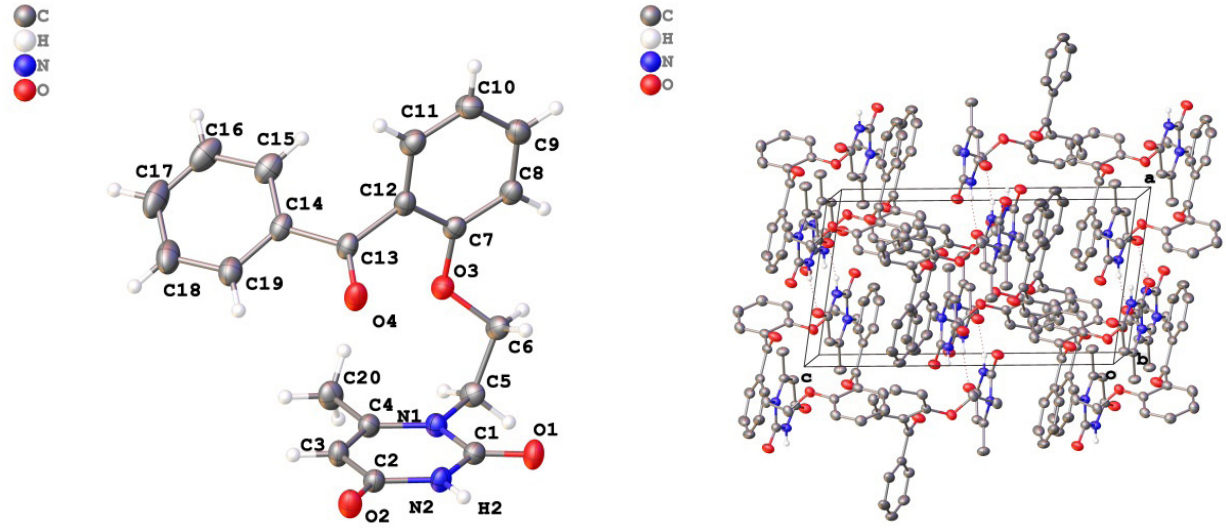

Figure 2 – Visualization of the 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil molecule

Note: (A) – General view of the molecule and (B) – Crystal packing

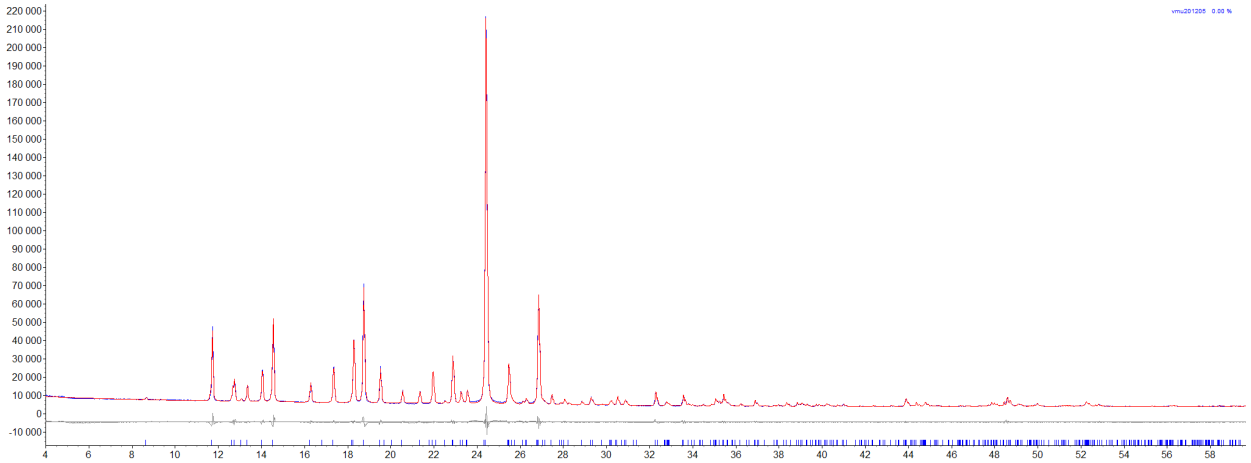

Figure 3 – General view of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil diffractogram (a blue line – experiment, a red line – calculation, a gray line – a difference curve)

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Table 1 – Basic crystallographic data and parameters of the structure refinement for 1-[2-(2-benzoylphenoxy) ethyl] -6-methyluracil

Gross formula	$C_{20}H_{18}N_2O_4$
Molecular mass	350.36
Temperature, K	120
Space group, Z	$P2_1/n$, 4
Cell parameters:	4
a, Å	8.1352(7)
b, Å	13.7868(11)
c, Å	15.0957(12)
a, °	90
b, °	98.443(2)
g, °	90
Cell volume, V, $Å^3$	1674.8(2)
Density, d_{calc}, g cm^{-3}	1.390
Absorption coefficient, m, cm^{-1}	0.98
Structure factor F (000)	736
Crystals size, mm	0.25 × 0.17 × 0.14
Crystalline form, color	Призмы, коричневый
$2q_{max}$,	61.36
Number of measured reflections	22276
Number of independent reflections	5160
Number of reflections with I>2s (I)	3182
Number of refined parameters [I>2s (I)]:	286
R_1	0.0511
wR_2	0.1258
GOF	1.000
Residual electron density, e·$Å^{-3}$(r_{min}/r_{max})	0.344/-0.212

Table 2 – Diffraction maxima of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil sample

Angle 2q, °	D-space (d), Å	Relative intensity	Angle 2q, °	D-space (d), Å	Relative intensity
4.706	18.7609	0.30%	35.156	2.55063	1.30%
8.676	10.18426	0.50%	35.439	2.53089	2.90%
11.735	7.53531	19.00%	36.223	2.47787	0.70%
12.765	6.92951	5.40%	36.892	2.43449	1.40%
13.074	6.76618	0.50%	37.367	2.4046	0.30%
13.358	6.62292	4.00%	37.799	2.37813	0.30%
14.048	6.29941	8.10%	37.967	2.368	0.50%
14.555	6.08082	21.20%	38.364	2.34439	1.00%
16.287	5.43779	5.20%	38.853	2.31603	1.00%
17.352	5.10653	9.30%	39.073	2.30348	0.90%
18.292	4.84624	16.30%	39.292	2.29113	0.50%
18.746	4.7297	30.70%	39.86	2.25981	0.40%
19.518	4.54444	9.50%	40.209	2.24098	0.70%
20.544	4.31971	3.60%	40.775	2.21116	0.20%
21.352	4.15813	3.10%	40.996	2.19974	0.60%
21.957	4.0448	8.10%	41.818	2.15838	0.10%
22.549	3.93992	0.50%	41.767	2.1609	0.10%
22.882	3.88337	12.00%	42.364	2.13184	0.30%
23.253	3.82218	3.00%	43.254	2.09001	0.10%
23.552	3.77445	3.10%	43.892	2.06109	1.90%
24.415	3.64283	100.00%	44.381	2.03952	1.00%
25.463	3.49533	10.20%	44.793	2.0217	1.10%
26.287	3.38752	1.30%	45.324	1.99923	0.20%
26.854	3.31735	27.00%	46.412	1.95488	0.30%

Angle 2q, °	D-space (d), Å	Relative intensity	Angle 2q, °	D-space (d), Å	Relative intensity
27.469	3.24438	2.30%	46.735	1.94213	0.10%
28.068	3.1765	1.30%	47.417	1.91577	0.10%
28.864	3.09073	1.00%	47.931	1.89643	0.40%
29.282	3.04751	2.10%	47.989	1.89426	0.80%
29.804	2.99533	0.20%	48.597	1.87199	2.10%
30.239	2.95321	1.20%	49.131	1.85285	0.50%
30.520	2.92669	2.40%	49.999	1.82271	0.70%
30.865	2.89478	1.50%	51.332	1.77848	0.30%
31.227	2.86196	0.10%	52.285	1.74826	0.90%
32.285	2.77059	3.60%	52.831	1.73149	0.50%
32.802	2.72812	1.10%	56.284	1.63318	0.30%
33.576	2.66693	2.60%	57.546	1.60032	0.10%
33.998	2.63483	0.40%	58.432	1.57815	0.10%
34.508	2.59705	0.50%	59.064	1.56277	0.20%
34.869	2.57093	0.40%			

Table 3 – Results of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil elemental analysis

Pharmaceutical substance	C,%(M±σ), n=2	H,%(M±σ), n=2	N,%(M±σ), n=2
1-[2-(2-benzoylphenoxy)ethyl]-6 methyluracil	68.22±0.08	5.24±0.03	7.81±0.04
Theoretical calculation	68.60	5.11	8.00

Table 4 – Characteristic maxima of absorption bands of a sample substance (in cm^{-1}) in the near and middle IR range of 8000–370 cm^{-1}

Pharmaceutical substance	Absorption maxima, cm^{-1}
1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil	4002.5; 3940.0; 3914.1; 3896.3; 3861.3; 3847.4; 3831.5; 3811.5; 3788.7; 3775.7; 3741.0; 3707.2; 3697.4; 3682.9; 3653.6; 3640.3; 3623.9; 3318.4; 3186.1; 3157.8; 3080.0; 3060.6; 3036.4; 3014.2; 2967.7; 2933.3; 2900.5; 2881.0; 2864.3; 2792.1; 2587.1; 2552.3; 2470.9; 2443.9; 2427.6; 2386.4; 2354.8; 2341.2; 2303.6; 2240.7; 2189.0; 2105.5; 2090.1; 2075.2; 2038.8; 2016.0; 1987.2; 1951.2; 1924.4; 1888.1; 1860.0; 1823.9; 1701.3; 1665.7; 1613.6; 1596.2; 1578.6; 1531.9; 1483.0; 1473.8; 1462.9; 1449.1; 1442.1; 1427.0; 1409.3; 1393.1; 1358.1; 1313.7; 1292.2; 1270.8; 1242.8; 1179.2; 1151.7; 1121.2; 1107.8; 1075.2; 1062.4; 1043.3; 1025.0; 996.0; 982.0; 971.9; 959.6; 944.6; 929.9; 894.2; 869.8; 852.2; 833.3; 807.7; 775.5; 766.4; 755.3; 731.4; 717.1; 704.2; 689.1; 634.1; 609.9; 567.6; 533.5; 509.0; 461.9; 435.9; 418.2; 376.7

Table 5 – Results of ^{1}H, ^{13}C NMR spectra analysis of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil

Pharmaceutical substance	Position	Group	Chemical shift, δ ^{1}H, ppm	Chemical shift, δ ^{13}C, ppm
1-[2-(2-benzoylphenoxy) ethyl] -6-methyluracil	1	Ar, α-CH	7.21 d	113.16 s
	2	Ar, β-CH	7.10 t	121.61 s
	3	Ar, β-CH	7.50 t	132.23 s
	4	Ar, α-CH	7,29 d	128.94 s
	5	Ar, ipso-C	–	155.71 s
	6	Ar, ipso-C	–	129.13 s
	7	C=O	–	196.05 s
	9	Ar, ipso-C	–	136.92 s
	10.14	Ar, α-CH	7.67 d	129.71 s
	11.13	Ar, β-CH	7.46 t	129.08 s
	12	Ar, γ-CH	7.60 t	134.07 s
	16	O-CH_2	4.18 t	66.25 s
	17	N-CH_2	3.85 t	43.47 s
	19	C=O	–	151.82 s
	20	NH	11.10 s	–
	21	C=O	–	162.76 s
	22	=CH	5.12 s	101.55 s
	23	=C	–	154.49 s
	26	CH_3	1.82 s	19.88 s

Note: t – triplet, s – singlet, d – doublet, k – quartet

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Nuclear magnetic resonance spectroscopy

The experimental work to determine the NMR spectra of the test substance was carried out on a BrukerAvance-IIIHD 500 NMR spectrometer (Bruker, Germany). A portion of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil, 20 mg in weight, was dissolved in 600 µL of deuterated dimethyl sulfoxide (DMSO-d6). The resulting solution without further processing by "asis" was placed in an NMR spectrometer for recording ^{1}H, ^{13}C, HC-HMQC and HC-HMBC spectra. The ^{1}H-NMR spectra were recorded at the operating frequency of 500.13 MHz, ^{13}C – at the operating frequency of 125.76 MHz. To confirm the structure of the carbon skeleton of the potential product, ^{13}C spectra were recorded in the phase-sensitive JMOD version (CH_3, CH – signals with negative phases, CH_2, C – signals with positive phases), as well as inverse heteronuclear correlations HC – HMQC (direct interactions C – H), HC – HMBC (long range interactions C – H). Assignment in the ^{13}C-JMOD spectrum was performed based on the analysis of two-dimensional inverse correlations HC-HMQC and HC-HMBC. To confirm the structure of the carbon skeleton, ^{13}C NMR spectra were recorded in the phase-sensitive version of JMOD (C, CH_2 signals were directed upward; CH, CH3 signals were directed downward), HC-HMQC, HCHMBC.

Investigation of the action mechanism *in vitro*

The study of the action mechanism of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil was carried out by evaluating the inhibitory activity against wild-type HIV-1 RT and its mutant forms corresponding to drug-resistant viral strains using radioactively labeled nucleotides. To express the wild-type heterodimer of HIV-1 reverse transcriptase, the cells of *E. coli* strain M15 [pRep4] (Qiagen, Germany) transformed with the p6HRT-PROT plasmid, were used. To obtain mutant forms of HIV-1 reverse transcriptase, the cells of *E. coli* strain Rosetta DE3 (Qiagen, Germany) transformed with the target plasmids, were used.

During the study of the inhibitory activity of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil against mutant forms of HIV-1 RT, a panel of HIV-1 RT mutant forms with amino acid substitutions L100I, K103N, V106A, Y181C, G190A was used: they are prevalent in patients with resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs), lead to resistance through a variety of mechanisms and are accepted in defining the resistance profile of new anti-HIV drugs. Additionally, the inhibitory activity of 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil against a double mutant with the two most common substitutions K103N and Y181C was investigated. The substance of the drug efavirenz was used as a positive control of the HIV-1 RT inhibitor. The inhibition constant for the substance was determined by the Dixon method, i. e., based on the dependence of the inverse rate of the enzymatic reaction in the presence of an inhibitor on its concentration.

RESULTS AND DISCUSSION

A general view of the molecule and its crystal packing are shown in Fig. 2. The main crystallographic data and refinement parameters are presented in Table 1.

The general view of the diffraction pattern is shown in Fig. 3, the main characteristics of the diffraction maxima are shown in Table 2.

Refinement of the divergence between the experimental and theoretical data showed that the sample corresponds to one crystalline phase of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil. No other crystalline phases have been found out.

The obtained data of the thermal analysis of the substance 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil are shown in Fig. 4–6.

The process of weight loss begins at the temperature of 25-50° C. The decrease in weight in this temperature range is about 1%, which is associated with the removal of residual solvents or moisture from the sample. In the temperature range of 184–227° C, the weight loss is about 3%, which is associated with the destruction of the crystalline hydrate. After 300° C, the thermal changes begin to occur with the substance, ending in thermo-oxidative destruction. On the DTG curve, the peak with a top at 203° C corresponds to the temperature at which crystalline hydrate water is removed. On the DTA curve, the endothermic peak with a top at 230° C corresponds to the sample melting point.

It has been established that crystallization of 1-[2-(2-benzoylphenoxy) ethyl] -6-methyluracil is observed at the temperature of 191° C (Fig. 5). The melting point and the fusing heat upon repeated heating are somewhat lower than on the first one, which is associated with different conditions for the formation of the crystalline phase.

An endothermic peak with a minimum at 195° C was recorded on the thermogram. It is associated with the removal of crystallization water. The melting point of the crystalline phase was 227° C (Fig. 6).

The elemental analysis results are presented in Table 3.

Based on the results obtained, shown in Table 3, it can be concluded that the content of the analytes of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil corresponds to the theoretical content of the analytes calculated on the basis of the gross formula $C_{20}H_{18}N_2O_4$.

The spectra of 1-[2-(2-benzoylphenoxy)ethyl] -6-methyluracil in the UV-visible range are shown in Fig.7, 8.

The spectra show an absorption maximum at 251.0 nm for the solution in THF and 254.5 nm for the solution in DMSO. In this case, the optical density of DMSO increases much faster than of THF; therefore, in the case of THF, the measurement from 210 nm is possible. To obtain a pronounced maximum absorption of the substance in the UV-visible range, the following measurement conditions are recommended: a solution in tetrahydrofuran of spectral purity, the concentration of 0.10 mg/ml, measurements in a quartz cuvette with an optical path length of 1.00 mm, a registration of the spectrum in the range of 210–900 nm, the background should be pure THF.

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Table 6 – Inhibitory activity of NNRTIs against RT of wild (WT) and mutant strains of HIV-1 *in vitro*

Back transcriptase	Inhibition constant (Ki, μM)	
	1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil	Efavirenz
Wild type	0,23 ± 0,04	0,011± 0,002
L100I	>20[a]	0,14± 0,01
K103N	>20[a]	0,52± 0,1
V106A	>20[a]	0,11± 0,01
Y181C	12 ± 2,4	0,053± 0,006
G190A	8,3 ± 0,4	0,091± 0,008
K103N/Y181C	>20[a]	0,52± 0,08

Note: [a]The test substance, 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil, inhibits mutant forms of HIV-1 RT L100I, K103N, V106A and the double mutant K103N / Y181C at the concentration of 20 μM with the efficiency of 38%, 33%, 22% and 35%, respectively. No increase in inhibition of these mutant forms of HIV-1 RT at higher concentrations of 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil was observed, which is most likely associated with the achievement of the solubility limit of the compound in the reaction mixture containing 10% DMSO

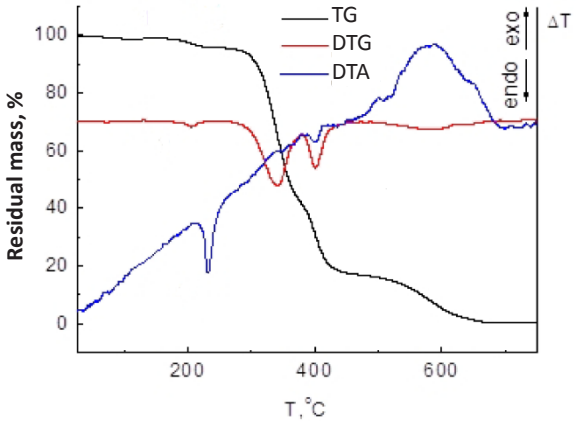

Figure 4 – Derivatogram of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil

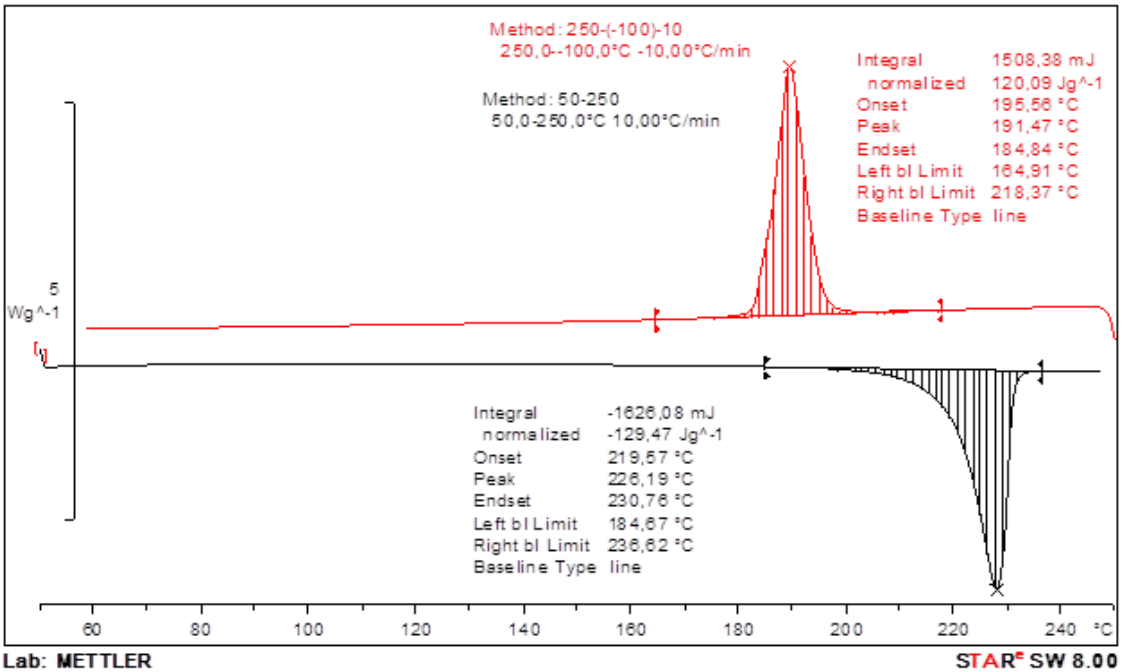

Figure 5 – DSC thermogram obtained by scanning 1- [2- (2-benzoylphenoxy) ethyl] -6-methyluracil

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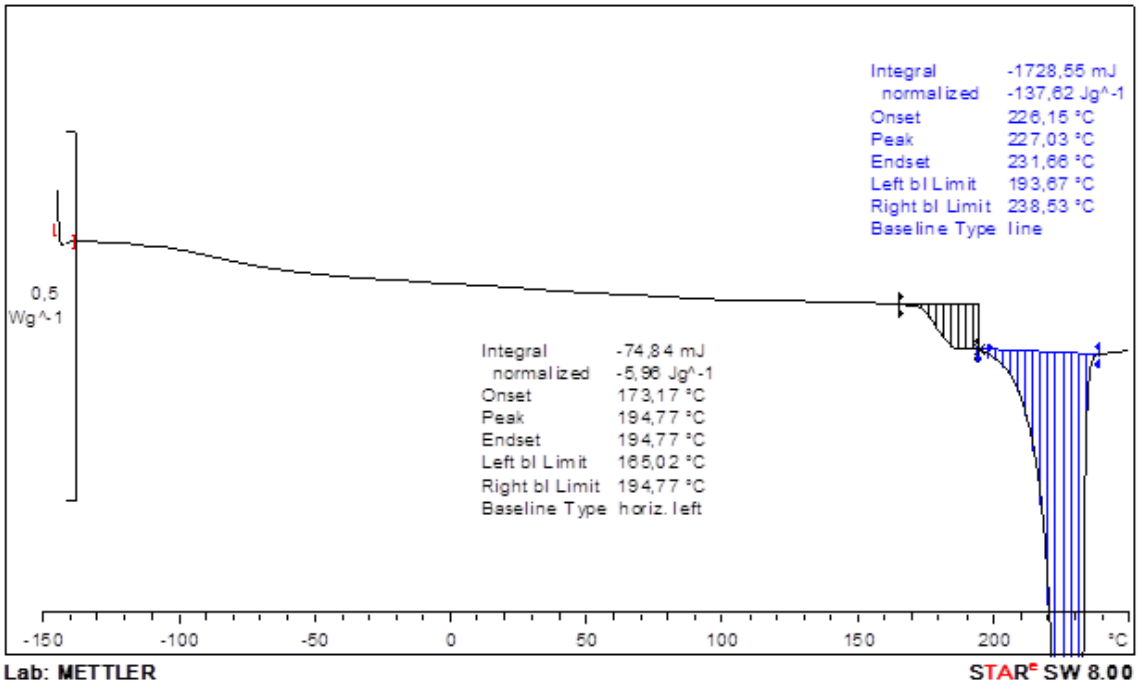

Figure 6 – DSC thermogram obtained by scanning 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil

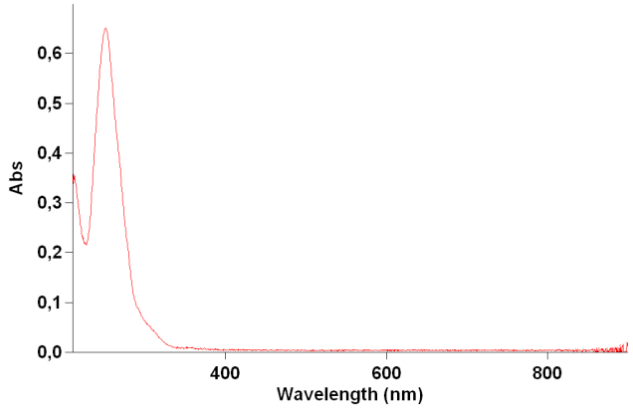

Figure 7 – Absorption spectrum of 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil tetrahydrofuran at the concentration of 0.1 mg/ml in the range of 210–900 nm in a cuvette with an optical path length of 1.00 mm

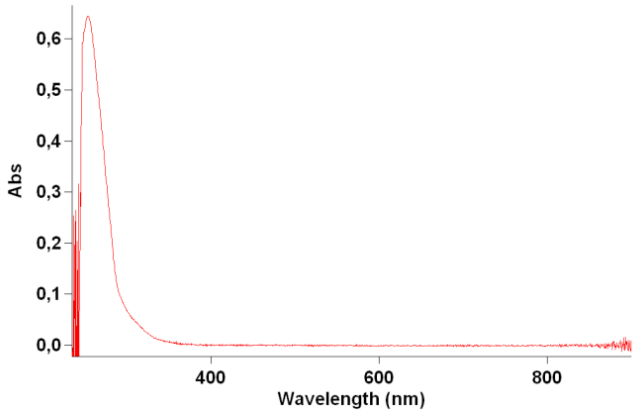

Figure 8 – Absorption spectrum of 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil dimethyl sulfoxide at the concentration of 0.1 mg/ml in the range of 235–900 nm in a cuvette with an optical path length of 1.00 mm

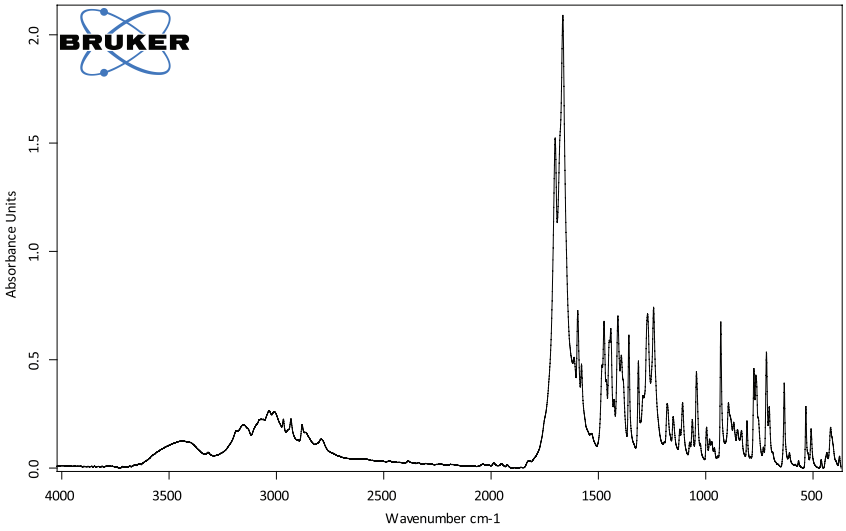

Figure 9 – IR absorption spectrum of 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracilav in the range of 4000-370 cm^{-1} in potassium bromide tablets

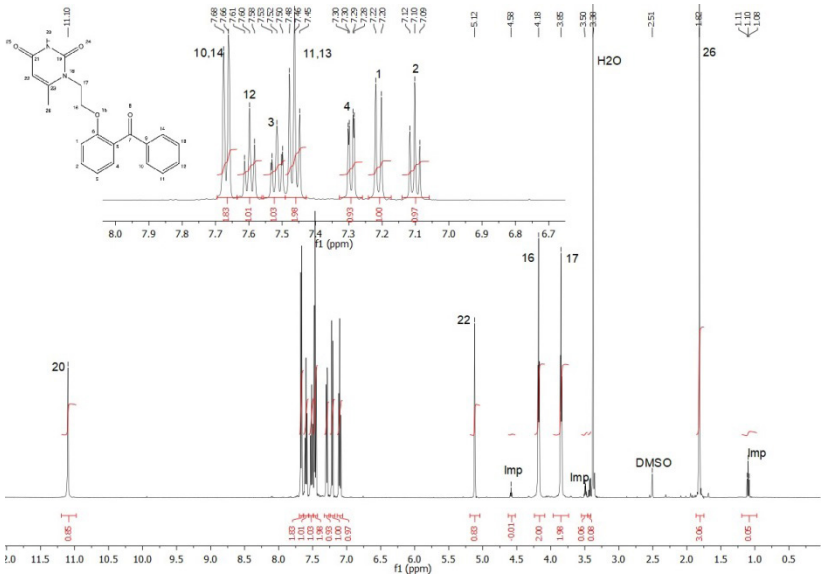

Figure 10 – 1H NMR spectrum of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil

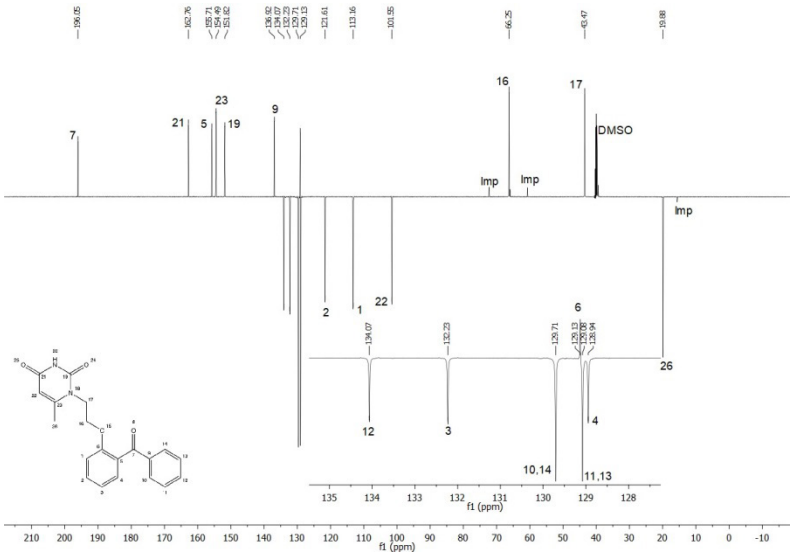

Figure 11 – ^{13}C NMR JMOD spectrum of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil

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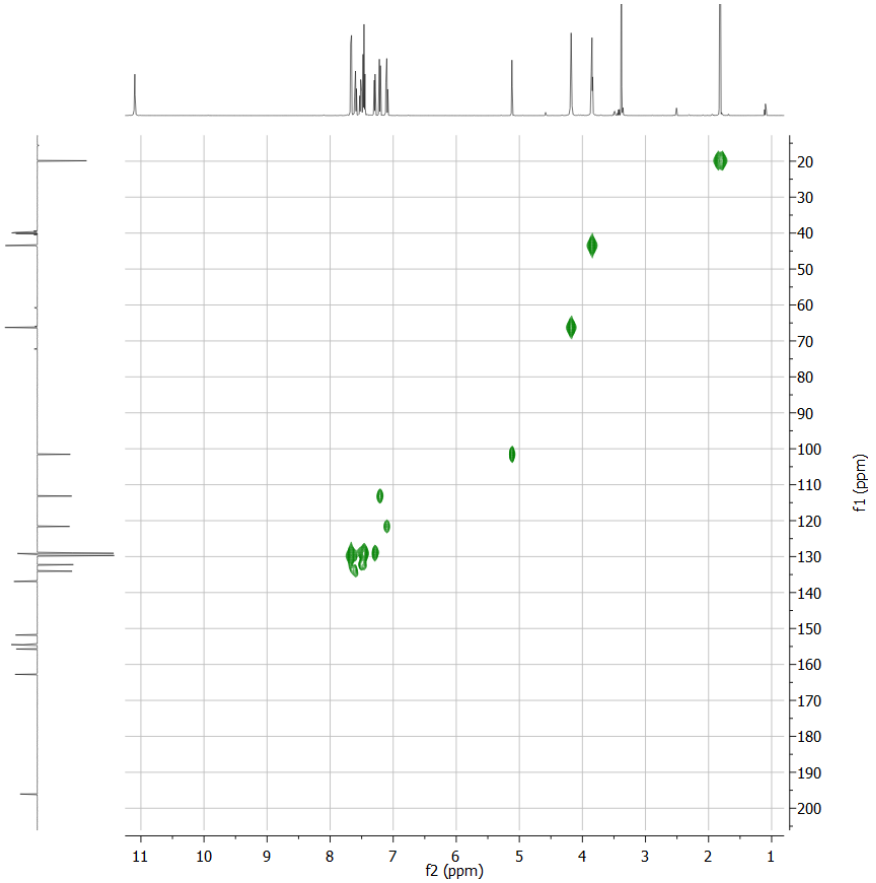

Figure 12 – NMR HC-HMQC spectrum of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil

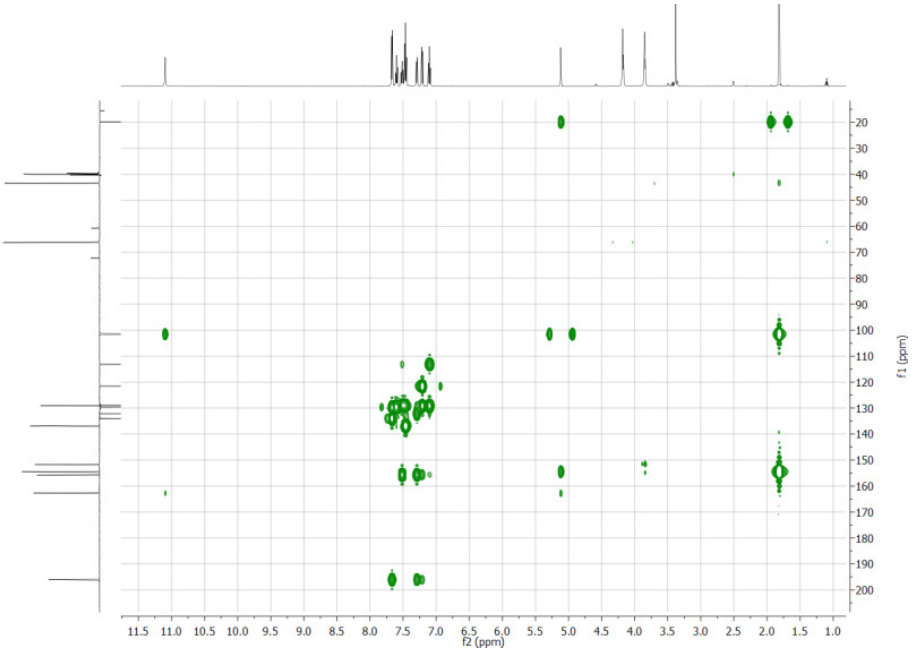

Figure 13 – HC-HMBC NMR spectrum of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil

Fig. 9 shows a general view of the IR absorption spectrum of the sample in potassium bromide tablets.

In the spectra of the sample in the IR range, there are many narrow and characteristic bands, which are summarized in Table 4. In addition, the spectrum of the sample in KBr in the area of 3400 cm – 1 contains a broad band corresponding to the moisture absorbed by potassium bromide; therefore, it was excluded from consideration.

Due to the complexity of the molecule, it is not possible to identify and unambiguously assign all signals to specific functional groups. Therefore, IR spectroscopy for a given molecule can be used as an additional one, along with NMR spectroscopy.

The obtained ^{1}H NMR spectrum of the analyte is shown in Fig. 10.

The most important characteristics of the NMR spectrum is its chemical shift (δ), which depends on the structure of the molecule. The electron density of protons in molecules is determined by the nature of the chemical bond and the induction effects of the surrounding groups, as a result of which the screening of protons becomes different and their signals appear in different areas of the spectrum.

The signals were assigned in the ^{1}H spectrum (Fig. 10). The spectrum shows that the sample contains signals related to both aliphatic fragments and aromatic rings. The signal of the NH group is also visible. In general, the range of the main product does not contradict the proposed structure. In the aromatic region, the spectrum of ^{1}H also contains low-intensity signals of impurities in the trace amounts, which, due to their low content, cannot be identified.

To confirm the structure of the carbon skeleton, ^{13}C NMR spectra were recorded in the phase-sensitive version of JMOD (C, CH_2 – signals upward, CH, CH_3 – signals downward), HC-HMQC, HCHMBC. These spectra are shown in Fig. 11–13.

The signals were assigned in the ^{13}C spectrum (Fig. 11). The spectrum shows that the sample contains signals related to both aliphatic fragments and positions in the aromatic rings of carbonyl groups. In general, the spectrum of 1- [2- (2-benzoylphenoxy) ethyl] -6-methyluracil does not contradict the proposed structure. The signals of minor impurities are also visible. Their content in the sample is below the detection limit of ^{13}C NMR spectroscopy.

Based on the interpretation results of the inverse two-dimensional correlation HC-HMQC and HC-HMBC spectra (Fig. 12,13), a complete assignment of signals in the ^{1}H and ^{13}C spectra was made, and the structure of the carbon skeleton product was confirmed. Fig. 12,13 also show that the distribution of two-dimensional correlation signals does not contradict the suggested structure of the proposed product. The results of assigning the bands to the functional groups of the molecules of the test substance are presented in Table 5.

It was found out that the number and a mutual arrangement of functional groups, the integral intensity of the signals in the ^{1}H spectrum corresponds to the structure of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil. The structure of the carbon skeleton corresponds to the structure of the test compound.

Modern instrumental methods of analysis make it possible to fully characterize a pharmaceutical substance, which is extremely necessary for its standardization, development of quality control methods and their subsequent inclusion in draft regulatory documents [26]. In this work, the results of X-ray, elemental, thermogravimetric, DSC analyzes, UV, IR and NMR spectroscopy of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil – the compound developed as a drug candidate for the HIV infection treatment – have been demonstrated.

The data obtained confirm the expected structure of the molecule: visualization of the molecule by an X-ray diffraction study, the gross formula $C_{20}H_{18}N_2O_4$ calculated by an elemental analysis, the mutual arrangement of functional groups and the structure of the carbon skeleton in the NMR spectrum correspond to the expected structure of the compound. The revealed characteristic peaks in the UV and IR spectra indicate the presence of basic functional groups characteristic of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil. The results of the X-ray phase study indicate the presence of one crystalline phase of the sample, which is consistent with the data of the thermal analysis methods, which have established the absence of substance polymorphs. During the NMR analysis, low-intensity signals of impurities in the trace amounts were detected, but their content was extremely low, which indicates the proper synthesis and a high degree of the substance purification.

The most important stage of the research was the study of the action mechanism of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil in relation to HIV-1 RT.

It was shown that 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil is an effective inhibitor of RT as a wild strain of HIV-1, and a number of its mutant forms (Table 6). The activity of the test object depends on mutations in the binding site of non-nucleoside RT inhibitors, acting by a non-competitive mechanism.

The results presented in Table 6 confirm that the object of the study belongs to the group of HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs). It should be noted that efavirenz inhibited an RT polymerase activity at lower concentrations than 1-[2-(2-benzoylphenoxy)ethyl] -6-methyluracil, with an equal antiviral activity *in vitro*.

A high variability of HIV leads to numerous substitutions of amino acids in RT, which form the resistance of the virus to NNRTIs [27]. According to the literature data [28], most of the identified mutations are localized in the hydrophobic pocket, the binding site of HIV NNRTIs, near the catalytic site of the enzyme. Among patients with resistance to NNRTIs, the most common mutations

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are K103N (56.98%) and Y181C (24.95%). Mutations G190A, L100I, V106A are less common (8.16%, 6.92% and 2.37%, respectively), but they also strongly affect the success of antiretroviral therapy, as they lead to the loss of the inhibitory activity of nevirapine by more than 2 orders of magnitude [29]. Mutations L100I, K103N, and Y181C are also characteristic of the patients with resistance to efavirenz and delaverdine [30]. Mutations Y181C and V106A are known to cause a drop in the activity of capravirin, a potential second-generation NNRTI withdrawn from clinical trials [31]. Lersivirin is an NNRTI based on capravirin. Having passed the clinical phase IIb, it has succeeded in achieving resistance to these mutations, but there was a decrease in its activity notified in the presence of the L100I mutation [32]. A moderate activity loss against the V106A mutation was characteristic of compound GW69564 [33], the structural modification of which made it possible to create another molecule (GW695634), which reached the III phase of clinical trials. Several mechanisms of RT resistance to the action of NNRTIs have been proposed: mutations L100I and G190A sterically prevent the placement of NNRTIs in the hydrophobic pocket [34], the Y181C mutation leads to the loss of interactions with amino acid residues inside the pocket [35], the K103N mutation makes it difficult for NNRTIs to enter the hydrophobic pocket [36, 37].

The work demonstrated the ability of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil to inhibit not only wild-type HIV-1 RT, but also its most common mutant forms with amino acid substitutions L100I, K103N, V106A, Y181C, G190A, widely present in NNRTI-resistant patients and leading to the resistance through a variety of mechanisms. These mutations are often studied when defining the resistance profile of new antiretroviral drugs. Additionally, the inhibitory activity of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil was shown against a double mutant with the two most common substitutions K103N and Y181C, which is extremely important for the development of a new antiretroviral drug aimed at overcoming the resistance of the virus. To confirm the activity of the compound against various strains and clinical isolates of infection, *in vitro* studies on the cell cultures infected with HIV, are required.

CONCLUSION

Thus, the results of the studies performed indicate that the UV spectrum of the compound has a pronounced absorption maximum when measuring a solution of the substance in tetrahydrofuran at the concentration of 0.10 mg / ml. In the IR spectrum there are specific bands in the range of 4000–370 cm^{-1}, which make it possible to use UV and IR spectra for the identification of the test compound in the substance. As a result of the performed X-ray structural, elemental, ^{1}H-NMR and ^{13}C-NMR analyzes, the structure of 1-[2-(2-benzoylphenoxy)ethyl]-6-methyluracil has been confirmed.

The possibility of using UV, IR and NMR spectroscopy, as well as thermal analyzes to confirm the authenticity of the substance during its verification, has been shown. The developed methods can be used in the quality control and are included in the draft regulatory document for the substance 1-[2-(2-benzoylphenoxy) ethyl]-6-methyluracil.

The studies of the action mechanism of the test substance against HIV-1 RT showed that the compound belongs to the HIV-1 NNRTI group.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

Ekaterina A. Jain (Korsakova) – research methodology, interpretation of results, text writing;
Dmitry V. Demchenko – statistical processing of results, text writing;
Alexander A. Ozerov, Vadim Yu. Balabanyan – working out the research concept and design;
Marina N. Makarova, Valery G. Makarov – interpretation and visualization of results

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