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Communication

(3-(1*H*-Indol-3-yl)-2-(7,8,12,13-tetraoxa-10-azaspiro[5.7] tridecan-10-yl)propanoic acid) with Cytotoxic Activity

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Abstract: An efficient method for the synthesis of 3-(1*H*-indol-3-yl)-2-(7,8,12,13-tetraoxa-10-azaspiro[5.7] tridecan-10-yl)propanoic acid) via condensation of 7,8,10,12,13-pentaoxaspiro[5.7]tridecane with tryptophan under the action of a catalyst based on $Sm(NO_3)_3 \cdot 6H_2O$ has been developed. A high cytotoxic activity of eight-membered azadiperoxide against tumor cells Jurkat, K562, U937, and HL60 was established. Additionally, this compound is an inducer of apoptosis and affects the cell cycle.

Keywords: aza-peroxides; 7,8,10,12,13-pentaoxaspiro[5.7]tridecane; tryptophan; catalysis; condensation; cytotoxic activity; apoptosis; cell cycle

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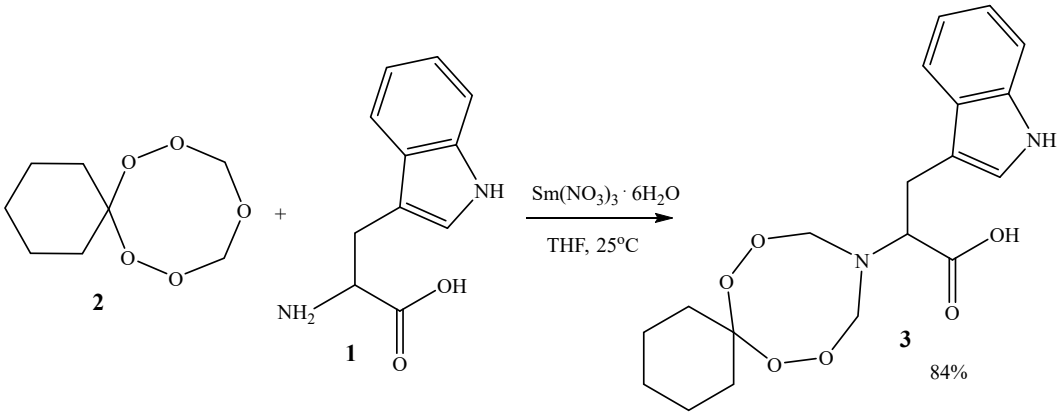
1. Introduction

Cyclic peroxides attract attention due to their antimalarial [1], antibacterial [2], and antitumor [3] activities. In a series of a large number of cyclic peroxides, heterocycloperoxides occupy a special place due to their higher biological activity [4]. It is known that the presence of heteroatomic substituents in cyclic peroxides causes antiparasitic, antitumor, antiviral, and antibacterial activities [4–6]. For example, artemisinin isolated from wormwood Artemisia annua containing an α-substituted peroxide moiety has antimalarial activity [7,8]. Similarly, natural verruculogen [5], dioxetanone [9], and other synthetic peroxides containing a nitrogen atom in the α-position relative to the peroxide group also exhibit high antimalarial activity [10]. There are literature data on the high cytotoxic activity of natural products with an oxygen or nitrogen atom adjacent to the peroxide group, such as 11-aza-artemisinin, 6-aza-artemisinin, and catharoseumine [10,11]. Methods for the synthesis of heteroatom-containing cyclic peroxides are limited. Recently [12–22], nitrogen-, sulfur-, and oxygen-containing cyclic di- and triperoxides with antitumor activity have been synthesized. The development of effective methods for the preparation of new heterocyclodi(tri)peroxides [12–22] contributes to the active search for new types of azaperoxides with high antitumor activity. Currently, much attention is paid to the study of stability, reactivity, pharmacokinetics, and the mechanism of action of biologically active peroxides, as well as the synthesis of new derivatives. Amino acids are a unique class of organic compounds. On the one hand, they are chiral compounds that are part of proteins and play an important role in biochemical processes in living organisms. On the other hand, the addition of pharmacophore groups to amino acids in some cases makes it possible to obtain drugs with high biological activity [23], and the use of amino acids as a transport function increases the selectivity of action and reduces the toxicity of drugs [24]. At the same time, the development of synthesis methods and the study of the properties of amino acid derivatives containing a peroxide fragment have not received sufficient attention. In this regard, we have synthesized a new azaperoxide derivative, 3-(1*H*-indol-3-yl)-2-(7,8,12,13-tetraoxa-10-azaspiro[5.7]tridecan-10-yl)propanoic acid), by the reaction recycling of 7,8,10,12,13-pentaoxaspiro[5.7]tridecane with the amino acid tryptophan. It is important to note here that tryptophan is an important mediator of anticancer immunity [25], so we studied the cytotoxic activity of the new 3-(1*H*-indol-3-yl)-2-(7,8,12,13-tetraoxa -10-azaspiro[5.7]tridecan-10-yl)propanoic acid).

2. Results and Discussion

It was found that during the interaction of tryptophan 1 with an equimolar amount of 7,8,10,12,13-pentaoxaspiro[5.7]tridecane **2** at ~20 °C, THF, 6 h using 5 mol. % catalyst $Sm(NO_3)_3 \cdot 6H_2O$ (3-(1*H*-indol-3-yl)-2-(7,8,12,13-tetraoxa-10-azaspiro[5.7]tridecan-10-yl)propanoic acid) **3** is formed with a yield of 84% (Scheme 1). In the absence of a catalyst, the reaction does not proceed. The choice of the catalyst is due to its high activity in the reactions of pentaoxaspiroalkanes with amino acids [16].



Scheme 1. Synthesis of compound **3**.

According to the data available in the literature [1–13,15,17–21], azaperoxides exhibit high biological activity. In this regard, the first obtained azadiperoxide 3 was investigated for cytotoxic activity against cell lines HL60, Jurkat, K562, U937 (Table 1).

Table 1. In vitro cytotoxic activity of azaperoxide **3** on tumor cell cultures (Jurkat, K562, U937, HL60) (μM).

	Jurkat (IC_{50}, μM)	**K562 (IC_{50}, μM)**	**U937 (IC_{50}, μM)**	**HL60 (IC_{50}, μM)**
Compound **3**	1.33 ± 0.17	1.08 ± 0.02943	1.21 ± 0.021	1.14 ± 0.011
Artemisinin	87.72 ± 7.26	90.43 ± 8.26	91.84 ± 8.44	94.23 ± 6.99

It was found that the synthesized diazatriperoxide **3** exhibits a cytotoxic effect on all selected tumor cell lines, and this compound exceeds the cytotoxicity of artemisinin by almost 90 times or more.

The effect of azadiperoxide **3** on apoptosis and the ability of this compound to influence the cell cycle was studied.

In Figure 1, it can be seen that this compound is an effective inducer of apoptosis by affecting the p53 deficient Jurkat cell line. After 24 h of incubation, almost 94% of all cells are in the stage of late apoptosis.

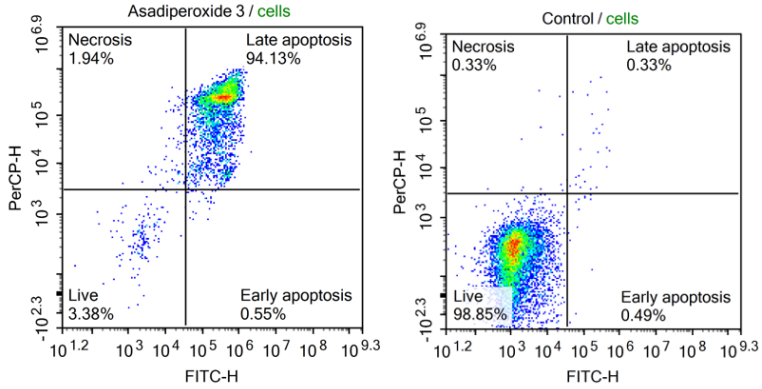


Figure 1. Cytofluorimetric study of apoptosis induction in Jurkat cells during incubation with azadiperoxide **3** at a concentration of 1.33 μM for 24 h. Annexine Alexa Fluor 488 and 7AAD staining.

Figure 2 shows the histograms of the cell cycle in the culture of cells treated with the newly synthesized diperoxide and the cells in the control sample. Azadiperoxide **3** effectively inhibits all phases of the cycle compared to untreated cells. We see a pronounced decrease in cell populations, both in the G1 phase and in the S and M/G2 phases, when compared with the control sample.

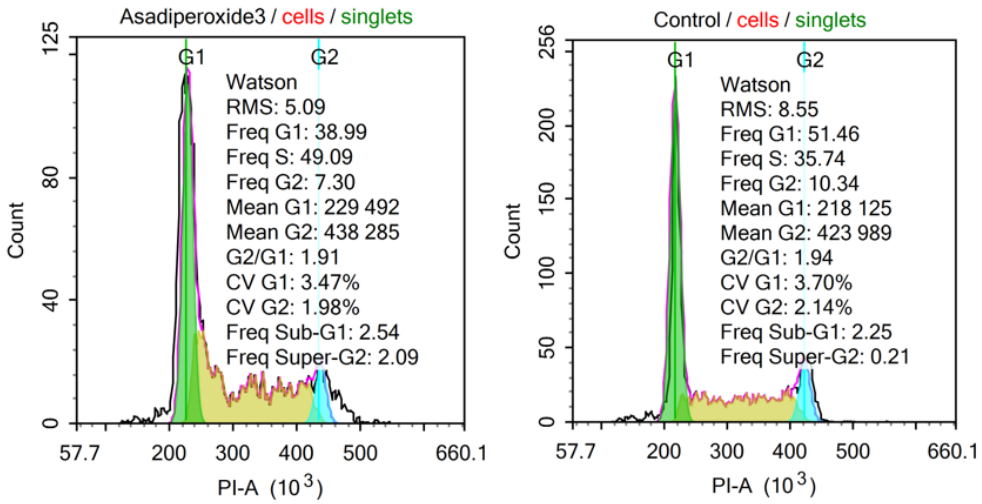


Figure 2. Cell cycle phases for Jurkat cells treated with azadiperoxide **3** at a concentration of 1.33 μM. The incubation time of compounds with cells was 48 h. Propidium iodide staining.

Thus, azadiperoxide **3**, which contains the methionine pharmacon in its structure, has a pronounced cytotoxic effect on tumor cells, which is 90 or more times greater than artemisinin, being an effective inducer of apoptosis and having a cytostatic effect due to an inhibitory effect on the cell cycle.

3. Materials and Methods

3.1. Chemistry

The reaction was performed at room temperature in air in round-bottom flasks equipped with a magnetic stir bar. The NMR spectra were recorded on a Bruker (Billerica, MA, USA) Avance 500 spectrometer at 500.17 MHz for ^{1}H and 125.78 MHz for ^{13}C according to standard Bruker procedures. $CDCl_3$ was used as the solvent, and tetramethylsilane, as the internal standard. Mass spectra were recorded on a Bruker Autoflex III MALDI TOF/TOF instrument with α-cyano-4-hydroxycinnamic acid as a matrix. Samples were prepared by the dried droplet method. The C, H, and N were quantified by a Carlo Erba 1108 analyzer. The progress of reactions was monitored by TLC on Sorbfil (PTSKh-AF-A) plates, with a 5:1 hexane: EtOAc mixture as the eluent and visualization with I2 vapor. For column chromatography, silica gel MACHEREY-NAGEL (0.063–0.2 mm) was used. The synthesis of the 7,8,10,12,13-pentaoxaspiro[5.7]tridecane **2** was as reported in the literature [22]. THF was freshly distilled over LiAlH4. Starting tryptophane was racemic.

Ring transformation reaction of 7,8,10,12,13-pentaoxaspiro[5.7]tridecane with tryptofane catalyzed by $Sm(NO_3)_3{\cdot}6H_2O$. General procedure. A round-botton flask mounted on a magnetic stirrer was charged with THF (5 mL), $Sm(NO_3)_3{\cdot}6H_2O$ (0.5 mmol), tryptofane (10 mmol), and 7,8,10,12,13-pentaoxaspiro[5.7]tridecane (10 mmol). The reaction mixture was stirred at room temperature (~25 °C) for 6 h, the solvent was evaporated, and the residue was chromatographed on a SiO_2 column to give (3-(1*H*-indol-3-yl)-2-(7,8,12,13-tetraoxa-10-azaspiro[5.7]tridecan-10-yl)propanoic acid) **3** as a pure compound.

(3-(1*H*-indol-3-yl)-2-(7,8,12,13-tetraoxa-10-azaspiro[5.7]tridecan-10-yl)propanoic acid) (**3**, Supplementary Materials).

Colorless oil; 0.31 g (84% yield), R_f 0.76 (PE/Et_2O = 10/1). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 1.40 (m, 1H, CH_2, *J* = 5 Hz), 1.58 (m, 2H, CH_2, *J* = 5 Hz), 1.68 (m, 1H, CH_2, *J* = 5 Hz), 1.80 (m, 6H, CH_2), 3.25 (dd, *J* = 10 Hz, 1H, CH_2), 3.36 (dd, *J* = 10 Hz, 1H, CH_2),

3.91 (t, J = 10 Hz, 1H, CH), 5.11–5.31 (m, 4H, CH_2), 6.96 (s, 1H, CH), 7.23 (m, 4H, CH), 9.54 (s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 22.4, 22.5, 22.6, 22.7, 25.2, 25.3, 25.6, 29.5, 29.8, 30.1, 30.5, 67.9, 85.6, 86.0, 109.3, 109.8, 112.2, 117.3, 119.2, 120.6, 121.5, 124.2, 127.2, 134.8, 173.5. MALDI TOF/TOF, m/z: 375 $[M - H]^+$. Anal.calcd. For $C_{19}H_{24}N_2O_6$: C, 60.63; H, 6.43; N, 7.44%. Found C, 60.61; H, 6.42; N, 7.43%.

3.2. Biological Screening

3.2.1. Cell Culturing

Cells (Jurkat, K562, U937, HL60) were purchased from the HPA Culture Collection (Salisbury, UK) and cultured according to standard human cell culture protocols. All cell lines used in this work were suspension cultures, and they were cultured in medium (RPMI, Gibco BRL) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37 °C in a humid medium containing 5% CO_2. The cells were then seeded into 24-well plates at 10 × 10^4 cells per well and incubated for 24 h with test compounds.

3.2.2. Cytotoxicity Assay

Cell viability was assessed by staining cells with 7-AAD (7-aminoactinomycin D) (Biolegend, San Diego, CA, USA). After incubation with the test compound, the cells were harvested, washed 2 times with PBS buffer, and centrifuged at 380× g for 6 min. The cell pellet was resuspended in 200 μL of flow cytometry staining buffer (PBS without Ca^{2+} and Mg^{2+}, 2.5% FBS) and stained with 6 μL of 7-AAD solution for 20 min at 37 °C in the dark. Samples were detected using the NovoCyte Penteon Flow Cytometer (ACEA) flow cytometry system.

3.2.3. Apoptosis Assay

Presented here is an apoptosis assay that allowed us to assess two markers of cell health: cell surface expression of phosphatidylserine and membrane permeabilization. Using reagents from the Millipore FlowCellect™ (Millipore, Bedford, MA, USA) Apoptosis Assay Kit provides information on early, mid, and late apoptosis with one simple assay. Cells were treated with synthesized compounds and incubated at 37 °C for 24 h. After this time, cells were dissociated with acutase solution, stained, and analyzed using flow cytometry (NovoCyte Penteon Flow Cytometer Systems, ACEA, San Diego, CA, USA) according to the protocols of the FlowCellect™ Apoptosis Assay Kit kit manufacturer.

3.2.4. Cell Cycle Assay

The cell cycle was analyzed with propidium iodide staining. After the cells were incubated with the test compound for 24 h, they were collected, washed 1–2 times with phosphate buffered saline (PBS), and centrifuged at 450× g for 5 min. The cells were then resuspended in 200 μL of a flow cytometry staining buffer (PBS without Ca^{2+} and Mg^{2+}, 2.5% FBS). At the next stage, the cells were transferred to 24-well plates with a density of 10 × 105 cells per well, then centrifuged at 450× g for 5 min, then fixed with cold ethanol 70% at 0 °C for 24 h. Then, the cells were washed from ethanol PBS buffer and incubated with 250 μL cell cycle detection reagent (Millipore) for 40 min at 22 °C. in the dark. The prepared samples were analyzed on the NovoCyte Penteon Flow Cytometer Systems (ACEA) flow cytometry system (ACEA, San Diego, CA, USA).

4. Conclusions

Thus, a new type of azaperoxide has been synthesized, namely (3-(1*H*-indol-3-yl)-2-(7,8,12,13-tetraoxa-10-azaspiro[5.7]tridecan-10-yl)propanoic acid), which has a high cytotoxic activity by the condensation reaction of 7,8,10,12,13-pentaoxaspiro[5.7]tridecane with tridecane under the action of $Sm(NO_3)_3{\cdot}6H_2O$. This azadiperoxide has a high cytotoxic activity against a number of suspension tumor cultures of hematological origin, exceeding the cytotoxic effect of artemisinin by more than 90-fold. At similar concentrations, the molecules also induces apoptosis in the p53-deficient Jurkat line and has a cytostatic

effect on cancer cells, reducing cell populations in all phases of the cell cycle. Thus, this compound is of considerable interest as a promising anticancer drug.

Supplementary Materials: The following are available online: ^{1}H NMR and ^{13}C NMR spectra of compound **3** [26].

Author Contributions: Conceptualization, U.M.D.; methodology and validation N.N.M. and I.R.I., performing the chemistry experiments; L.U.D. and U.M.D. performing the biology experiments; The manuscript was prepared through the contributions N.N.M., L.U.D., and U.M.D. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

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