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SYNTHESIS, CYTOTOXICITY AND ANTIBACTERIAL ACTIVITY OF 3-AMINO-9'-FLUORENESPIRO-5-HYDANTOIN

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Abstract

This paper presents a method for synthesis, cytotoxicity and antibacterial activity of 3-amino-9'-fluorenespiro-5-hydantoin. The structure of the obtained product was verified by UV-Vis, IR, FT-IR ATR, Raman, ^{1}H- and ^{13}C-NMR spectroscopy. In the present study, we have examined cytotoxic effect of 3-amino-9'-fluorenespiro-5-hydantoin on the retinoblastoma cell line WERI-Rb-1 and antibacterial activity against both Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* bacteria and the yeasts *Candida albicans*.

Key words: fluorenylspirohydantoins, 3-amino-9'-fluorenespiro-5-hydantoin, cytotoxic/antiproliferative effects, antibacterial activity

Introduction. The immense scientific interest in hydantoins (imidazolidines) and their derivatives stems from their well-known physiological activity and different medical and clinical applications, among which the most famous one is the use as antiepileptic drugs. Some spirohydantoins are known as aldose reductase inhibitors [1–5], which makes them useful in treatment of diabetes caused abnormalities. They have antitumour [6, 7], anticonvulsant, antiepileptic [8] and antiarrhythmic activity as well [9] and are used in the treatment of asthma. The inhibiting activity of spirohydantoins against muscle and liver glycogen phosphorylases is also well known [10]. The interest in the study of (9'-fluorene)-spiro-5-hydantoin /spiro-(fluorene-9,4'-imidazolidine)-2',5'-dione/ and its derivatives is mainly due to their biological activity. On the other hand, fluorene derivatives attract attention due to their luminescent and electroluminescent properties, caused

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by the inter- and intra-molecular charge distribution. The most powerful organic (and polymeric) light emitting diodes (OLED) are based on fluorene-containing compounds [11, 12]. Recently, we undertook a systematic study on the synthesis [13], structure [14] and complexation properties [15–17] of various spirodithiohydantoins (imidazolidine-2,4-dithiones), as well as on their complexes with copper, nickel and platinum.

The aim of this study is to present a method for synthesis of 3-amino-9'-fluorenespiro-5-hydantoin (3-AFSH), its structural elucidation and to examine its cytotoxic and antimicrobial activity.

Experimental. All used chemicals were purchased from Merck and Sigma-Aldrich. Melting point temperature of 3-amino-9'-fluorenespiro-5-hydantoin was determined by a SMP-10 digital melting point apparatus. The purity of the compound was checked by thin layer chromatography on Kieselgel 60 F_{254}, 0.2 mm Merck plate, eluent system (vol. ratio): benzene : acetone = 5 : 1. Electronic spectrum was measured on a Lambda 9 Perkin-Elmer UV/Vis/NIR Spectrophotometer from 200 nm to 1000 nm. The IR spectrum of 3-AFSH was registered in KBr pellet on a Bruker FT-IR VERTEX 70 spectrometer from 4000 cm^{-1} to 400 cm^{-1} at resolution 2 cm^{-1} with 25 scans. Attenuated Total Reflection FTIR (ATR) spectrum was registered on the same instrument by ATR accessory MIRacle™ with a one-reflection ZnSe element (Pike) and the stirred crystals of 3-AFSH were pressed by an anvil to the reflection element; the spectrum was from 4500 cm^{-1} to 600 cm^{-1} at resolution 2 cm^{-1} with 16 scans. The Raman spectrum of 3-AFSH (the stirred crystals placed in aluminium disc) was measured on a RAM II (Bruker Optics) with a focused laser beam of 200 mW power of Nd:YAG laser (1064 nm) from 4000 cm^{-1} to 51 cm^{-1} at resolution 2 cm^{-1} with 25 scans. The NMR spectra were taken on a Bruker Avance II+ 600MHz NMR spectrometer operating at 600.130 and 150.903 MHz for ^{1}H- and ^{13}C-, respectively, using the standard Bruker software. Chemical shifts were referenced to tetramethylsilane (TMS). Measurements were carried out at ambient temperature.

The synthesis of the target compound, 3-amino-9'-fluorenespiro-5-hydantoin (3), was carried out in accordance with Figure 1. The 9'-fluorenespiro-5-hydantoin (2) was obtained by an interaction of 9*H*-fluoren-9-one (1) with sodium cyanide

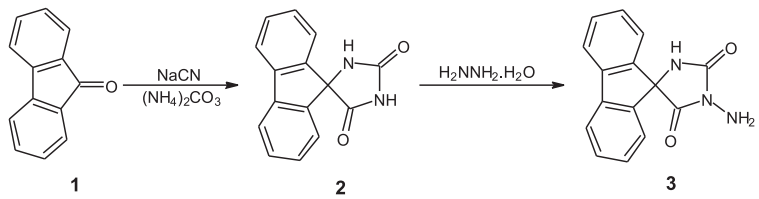


Fig. 1. Synthesis of 3-amino-9'-fluorenespiro-5-hydantoin

and ammonium carbonate, following a method described by NAGASAWA et al. [18].

Synthesis of 3-amino-9'-fluorenespiro-5-hydantoin (3-AFSH). A mixture of 7.5 g (0.03 mol) of 9'-fluorenespiro-5-hydantoin (2) and 15 ml of hydrazine hydrate was refluxed for five hours. After cooling down to room temperature, the reaction mixture was poured onto small quantity of crushed ice and was left overnight. The crystalline product obtained (3) was filtered off and recrystallised from ethanol.

Yield: 7.0 g (88%), T.t. = 334–335 °C, R_f = 0.76 (benzene : acetone = 5 : 1)

The spectral data of the 3-amino-9'-fluorenespiro-5-hydantoin are as follows:

UV-Vis (DMSO): λ_{max} = 306 nm, 295 nm, 271 nm.

IR (ν_{max}, cm^{-1}): 3312, 3274, 3096, 3067, 2918, 1690, 1642, 1559, 1514, 1476, 1452, 1321, 1303, 1230, 1199, 1180, 1152, 1107, 1094, 1059, 1028, 975, 955, 886, 847, 746, 737, 653, 631, 623, 603, 567, 556, 519, 435, 418 cm^{-1}.

ATR (ν_{max}, cm^{-1}): 3359, 3315, 3270, 3096, 3066, 2916, 1686, 1638, 1566, 1508, 1476, 1451, 1321, 1301, 1251, 1229, 1197, 1179, 1151, 1108, 1094, 1053, 1028, 975, 957, 946, 886, 847, 786, 754, 736, 707, 681, 653, 630, 622 cm^{-1}.

Raman (ν_{max}, cm^{-1}): 3053, 2924, 1697, 1610, 1583, 1485, 1449, 1353, 1293, 1230, 1170, 1152, 1125, 1054, 1021, 971, 888, 781, 744, 718, 654, 514, 441, 418 cm^{-1}.

^{1}H NMR (DMSO-d_6) δ/ppm: 8.60 (s, 1H, NH), 7.87 (d, J = 7.5 Hz, 2H), 7.57 (d, J = 7.4 Hz, 2H), 7.44 (t, J = 7.4 Hz, 2H), 7.34 (td, J = 7.4, 1.0 Hz, 2H).

^{13}C NMR (DMSO-d_6) δ/ppm: 172.09 (C=O), 157.10 (C=O), 145.96 (2×C), 140.33 (2×C), 129.15 (2×CH), 128.33 (2×CH), 125.27 (2×CH), 120.81 (2×CH), 55.46 (spiro).

Biological assay. ***Cell line.*** The retinoblastoma cell line (WERI-Rb1) was purchased from ATCC-HTB-169. It was cultured in RPMI 1640, containing 10% FCS, streptomycin and penicillin at 37 °C in a humidified atmosphere containing 5% CO_2.

WST-1 assay and cytotoxicity. The 3-amino-9'-fluorenespiro-5-hydantoin solved in 100% DMSO was diluted in RPMI-1640 culture medium containing penicillin and streptomycin and brought to a concentration of 200 µM. The concentration of DMSO was thus decreased to 0.2% which did not itself influence cell viability. The cytotoxic effect of 3-amino-9'-fluorenespiro-5-hydantoin was determined on the retinoblastoma cell line WERI-Rb-1 using Cell Proliferation Reagent WST-1 (Roche Applied Science). Cells were seeded in triplicates at a density of 6.5 × 10^4 cells/well in a 96-well plate. After a cultivation period of 24 h at 37 °C and 5% CO_2, 100 µl of the compound solution were added to the wells making up a final volume of 200 µl. Cells grown in culture medium alone or with appropriate concentrations of DMSO were used as controls. The cytotoxic effect of the compound was measured at three different time points – after 24 h, 48 h and 72 h. At each time point WST-1 (Water soluble Tetrazolium salts) reagent was added to the cells (1:10 final dilution). After an incubation period

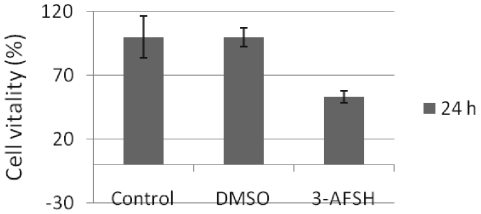
of 4 h at 37 °C and 5% CO_2 the absorbance was measured using a microplate ELISA SUNRISE reader at a wavelength of 450 nm with a reference filter at 620 nm. The percentage cell proliferation was calculated as a ratio of the sample OD (optical density) value to the control OD value. Each experiment was repeated 3 independent times and the results were presented as mean ± standard deviation of the mean.

Antimicrobial assay. The antimicrobial effect of 3-amino-9'-fluorenespiro-5-hydantoin against clinically isolated Gram-positive, Gram-negative bacteria – *Staphylococcus aureus*, *Escherichia coli* and the yeasts *Candida albicans* was studied. For this purpose the method described by IVANOV was used [19]. The substance was dissolved in 15% DMSO solution and added to growing media in double fold decreasing concentrations between 50 – 0.012 ppm. 200 µl of each dilution were inoculated in an amount of 1×10^6 CFU (Colony-forming unit) for *E. coli*, 1×10^6 CFU for *S.aureus* and 1×10^6 CFU for *C. albicans* and transferred in microplates. The plates were cultivated at 37 °C for 18–20 h and 48 h for the bacteria and the yeast, respectively. Two growing media were used for the cultivation of the test microorganisms – Muller-Hinton broth for the bacteria and Sabouraud broth for the yeast. The optical density of the samples was read at the end of the cultivation period with ($\lambda = 600$ nm) against inoculated media. The results were presented through samples turbidity.

Results and discussion. 3-AFSH was investigated by electronic UV-Vis, IR, Raman, ^{1}H- and ^{13}C-NMR spectroscopy. Maxima in the electronic spectrum of the 3-amino-9'-fluorenespiro-5-hydantoin were observed at 306 nm, 295 nm and 271 nm. The IR bands at 3312 cm^{-1} and 3274 cm^{-1} of 3-AFSH that were observed may refer to the stretching vibrations of the two N-H groups of the hydantoin ring. The two vibrational (N^1-H) and (N^3-NH_2) stretching modes did not appear in the Raman spectrum. The C=O stretching vibration gives rise to strong IR bands and weak Raman bands [20]. The carbonyl C=O stretching band was easily identified in the IR spectrum because of its intensity and its lack of interference with most other group frequencies. In the IR spectrum of 3-AFSH the bands at 1690 cm^{-1} and 1642 cm^{-1} can be attributed to stretching vibrations of C^4=O and C^2=O groups of the hydantoin ring. In Raman spectrum of 3-AFSH appears only one band at 1697 cm^{-1}, ν (C^4=O), with a very low intensity. Several bands in the Raman spectrum (3065, 3053, 3042 cm^{-1}) and in the IR spectrum (3096, 3067 cm^{-1}) were for stretching vibrations of CH in fluorene moiety.

Due to the extremely low water solubility of the 3-AFSH the highest concentration that could be achieved for our experiment was 100 µM. The results from the cytotoxicity assay on the human WERI-Rb-1 cell line showed that 3-amino-9'-fluorenespiro-5-hydantoin reduced the number of cells by around 50% after 24 h (Fig. 2). However, the cytotoxicity of this compound displayed no increasing effect after longer incubation times. On the contrary, cells vitality decreased only by 25% after 48 h and did not change at all after 72 h (Table 1).

Fig. 2. Decrease in cells vitality after treatment with 3-amino-9'-fluorenespiro-5-hydantoin for 24 h. Treatment with 1% DMSO did not show any cytotoxic effect compared to the control with culture medium, whereas 3-AFSH led to a 50% reduction in cell number



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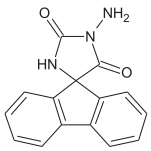
Percentage of viable cells (retinoblastoma cell line WERI-Rb-1) after treatment with 3-amino-9'-fluorenespiro-5-hydantoin for 24 h, 48 h and 72 h

compound	24 h	48 h	72 h
 *3-amino-9'-fluorenespiro-5-hydantoin*	52.98%	74.03%	100%

The results of our study showed that 3-AFSH had only a short-term effect on cancer cells viability. Longer incubation periods with this substance did not influence cell numbers. The exact reasons for this are still to be elucidated, however, substance instability could be a possible explanation. Based on our preliminary investigations we can conclude that 3-AFSH does not show a strong potential as a therapeutic drug against cancer.

The 3-amino-9'-fluorenespiro-5-hydantoin showed pronounced antimicrobial activity against the bacteria *Escherichia coli* (MIC $\geq$ 50 µM). There was no activity towards *Staphylococcus aureus* and *Candida albicans*.

Conclusions. The synthesis of 3-amino-9'-fluorenespiro-5-hydantoin was described and the various spectral data, UV-Vis, IR, FT-IR ATR, ^{1}H-, ^{13}C-NMR and Raman spectroscopy confirmed its structure. The preliminary results of our study revealed that 3-AFSH could not serve as potential anticancer agent. The results for 3-ASFH showed pronounced antimicrobial activity against the bacteria *Escherichia coli* and no activity towards *Staphylococcus aureus* and *Candida albicans.*

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