SYNTHESIS, CHARACTERIZATION, *IN VITRO* ANTIPROLIFERATIVE AND ANTIMICROBIAL STUDY OF 3-METHYL-(9'-FLUORENE)-SPIRO-5-(2,4-DITHIOHYDANTOIN)

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ABSTRACT

This paper presents a method for synthesis, *in vitro* antiproliferative and antibacterial study of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin). The structure of the obtained product was investigated by UV-Vis, IR, FT-IR ATR and Raman spectroscopy. The anticancer effect of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) was determined in both adherent and suspension cell lines originating from tumors in humans (A2058, ATCC and WERI-Rb-1). The cytotoxic effect was evaluated by WST-assay (Roche Applied Science). The antimicrobial activity of fluorenylspirohydantoin derivative against clinically isolated Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella enterica subsp enterica* ATCC BAA-2162 and *Pseudomonas aeruginosa* ATCC 9027) and the yeast *Candida albicans* ATCC 10231 was also studied.

Key words: *3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin), cytotoxic effect, antimicrobial activity.*

INTRODUCTION

The hydantoins (imidazolidines) and their derivatives are a class of organic compounds with a wide range of applications in various fields. Their antiepileptic, anticonvulsant and antitumor effect (Suzen et al., 2000; Rodgers et al., 1977), as well as capability to inhibit aldose reductase activity (USA Patent, 1989; Sarges et al., 1988) and successful application in diabet treatment were well discussed. Thioanalogues of hydantoins are also of interest, and their structure and biological activities were also investigated (Rydzik et al., 1978; Rydzik et al., 1979). Recently, we undertook a systematic study on the synthesis (Marinov et al., 2005), structure (Shivachev et al., 2006; Ahmedova et al., 2009) and complexation properties (Ahmedova et al., 2008; Ahmedova et al., 2010; Ahmedova et al., 2008) of various dithiohydantoins (imidazolidine-2,4-dithiones) as well as on their complexes with copper, nickel and platinum. Fluorene derivatives attract attention due to their luminescent and electroluminescent properties, caused by the inter- and intra-molecular charge distribution. The most powerful organic light emitting diodes (OLED) are based on fluorene-containing compounds (Thomas et al., 2006; Al Attar et al., 2005). Recently, we reported a method for synthesis, cytotoxicity and antibacterial activity of 4'-bromo-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) and new complexes of (9'-fluorene)-spiro-5-hydantoin and (9'-fluorene)-spiro-5-(2-thiohydantoin) with Pt(II) (Marinova et al., 2013 Marinova et al., in press).

That is why, the aim of this work is to present a method for synthesis of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin), its structural elucidation and to examine its cytotoxic and antimicrobial effects.

EXPERIMENTAL

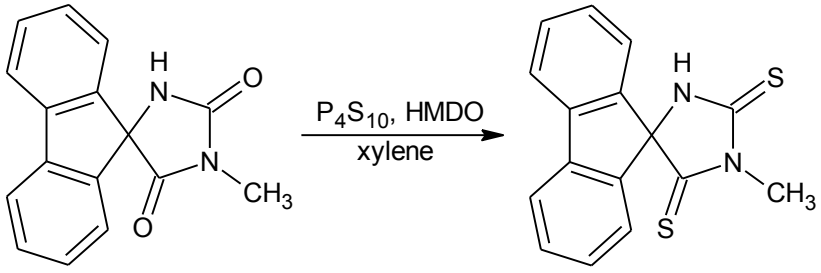
Instrumentation and methods

All used chemicals were purchased from Merck and Sigma-Aldrich. Melting point temperature of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) 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 plates, eluent system (vol. ratio): benzene : ethanol = 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-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin 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); the stirred crystals of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin 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-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin (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.

Synthesis of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin)

A mixture of 7.92 g (0.03 mol) of 3-methyl-(9'-fluorene)-spiro-5-hydantoin (1), 4.89 g (0.011 mol) of P_4S_{10}, 21 ml (0.1 mol) of hexamethyldisiloxane and 100 ml of xylene was refluxed for 4 hours. The crystalline product obtained (2) was filtered off and was recrystallized from methanol/water solution.

Yield: 6.35 g (75 %), M. p. = 282-283 ^oC, R_f = 0.81 (benzene : ethanol = 5 : 1).



1 **2**

Scheme 1. *Synthesis of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin)*

UV-Vis (DMSO): λ_{max} = 287, 278, 257 nm.

IR (KBr, cm^{-1}): 3250, 3081, 2738, 1965, 1924, 1602, 1586, 1525,1474, 1449, 1424, 1375, 1318, 1283, 1236, 1216, 1197, 1142, 1102, 1045, 1031, 1005, 983, 948, 935, 925, 911, 873, 863, 819, 784, 767, 755, 744, 730.

Relative Raman intensity, ν_{max}: 3049, 1606, 1485, 1450, 1358, 1297, 1218, 1152, 1109, 1021, 986, 745, 698, 683, 512, 458, 418 cm^{-1}.

FT-IR ATR, ν_{max}: 3281, 3106, 3040, 2958, 2735, 1602, 1520, 1448, 1415, 1373,1318, 1284, 1237, 1216, 1195, 1135, 1102, 1046, 1030, 1005, 984, 934, 926, 913, 863, 820, 784, 760, 753, 744, 726, 681, 657, 635, 619 cm^{-1}.

WST-1 cell proliferation assay

The cytotoxic effect of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) was assessed on a suspension and an adherent cell lines using WST-1 assay (Cat. No11 644 807 001, Roche). The suspension cells (WERI-Rb1) were cultured in RPMI 1640 medium, the adherent cells (A2058) – in DMEM. Both cell media contained 10% FCS, 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were cultured at 37 ºC in a humidified atmosphere containing 5% CO_2.

The compound was first dissolved in DMSO and then diluted in the respective culture medium. The concentration of DMSO in the wells did not exceed 1%. Cells were seeded in triplicates in 96-well plates at a density of $6{,}5x10^4$ cells/well (WERI-Rb1) and $2x10^5$ cells/well (A2058). After a cultivation period of 24h, the compound was added at a concentration 500μM on WERI-Rb1 cells and 50, 100, 250, 500 μM on A2058 cells, and incubated for 24, 48 and 72h respectively. WST-1 was added to the cells at these time points and incubated for 4h.

After an incubation period 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 of viable cells was calculated as a ratio of the OD value of the sample to the OD value of the control. The data are presented as mean ± standard deviation of the mean.

Antimicrobial assay

The antimicrobial effect of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) against Gram-positive bacteria - *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis*, Gram-negative bacteria – *Escherichia coli* ATCC 25922, *Salmonella enterica subsp enterica* ATCC BAA-2162, *Pseudomonas aeruginosa* ATCC 9027 and the yeasts *Candida albicans* ATCC 10231 was studied according to the agar diffusion method. Melted PCA (Scharlau) nutrient medium was inoculated through the addition of 1 cm^3 of microbial suspension ($1x10^{10}$ CFU/cm^3 for the bacteria and $1x10^9$ CFU/cm^3 for the yeats) and was poured in Petri dishes - 20 cm^3 in each dish. Wells with 7 mm diameter were made in the solidified and cooled agar medium. 50 μl of the tested substance solution (6 mg/cm^3 in 15% DMSO) was pipetted into the wells. The Petri dishes were incubated at 37ºC for 24-48 h. The inhibition zone was measured. Zones with diameter more than 7 mm were considered zones of inhibition.

RESULTS AND DISCUSSION

The synthesis of the 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) was carried out in accordance to ***Scheme 1***. The compound **2** was investigated by electronic UV-Vis, IR, FT-IR ATR and Raman spectroscopy. Maxima in the electronic spectrum of the 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) were observed at 287 nm, 278 nm and 257 nm. The IR bands at 3250 cm^{-1} and 3081 cm^{-1} of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) that were observed may refer to the stretching vibrations of the two N-H groups of the hydantoin ring. The one of two vibrational (N^1-H) and (N^3-H) stretching modes did not appear in the Raman spectrum. In the IR spectrum of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) the bands at 1602 cm^{-1} and 1586 cm^{-1} can be attributed to stretching vibrations of the two C=S groups of the hydantoin ring. In Raman spectrum of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) the one of the two C=S groups was appeared at 1606 cm^{-1}. The other vibrational (C=S) stretching modes did not appear in the Raman spectrum.

The effect of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) on the proliferation of WERI-Rb1 cells after 24 and 48 hours of treatment is presented in ***Figure 1***. Our results showed a relatively weak cytotoxic effect of the studied substance. It led to a reduction in the number of viable cells only by 15-20% after 24h. The longer incubation time did not seem to affect cell vitality. In contrast, we detected an increase in cell number which can be explained by the lack of long-term cytotoxic effect of the organic compound.

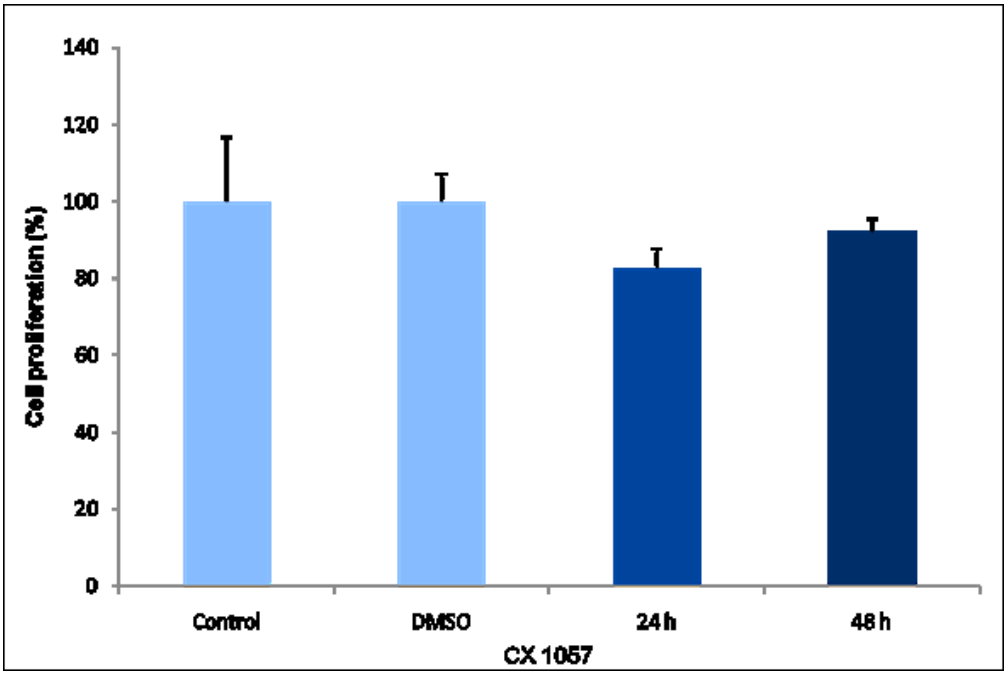


Figure 1. *Effect of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin (500 μM) on the proliferation of WERI-Rb1 after 24 h and 48 h of treatment*

We performed the same test on another cell line – the melanoma cell line A2058. We did not observe any effect of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) on cell proliferation in contrast to the data we obtained for the WERI-Rb1 cell line. The results from both cell lines suggest that the studied substance has low cytotoxic potential which makes it inappropriate as an anticancer agent.

The results for the antimicrobial activity of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) are presented in ***Table 1***. It showed no antimicrobial activity against the Gram negative bacteria *Escherichia coli* and *Salmonella enterica subsp enterica.* The activity against *Pseudomonas aeruginosa* and *Candida albicans* was low (inhibition zones of 9 and 11 mm, respectively). There was a strong inhibitory effect towards the Gram positive *Bacillus subtilis, Staphylococcus aureus*. The presence of single cell colonies in the inhibition zone for *Bacillus subtilis* shows that there are cell with different sensitivity towards this substance within the strain.

Table 1. Antimicrobial activity of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin)

№	Test microorganism	Viable cells count in the nutrient medium, cfu/cm^3	Inhibition zone, mm
1	*Escherichia coli* ATCC 25922	1,02.10^9	-
2	*Salmonella enterica subsp enterica* ATCC BAA-2162	1,12.10^9	-
3	*Bacillus subtilis*	2,21.10^9	13 25*

Table 1. Antimicrobial activity (continues)

4	*Staphylococcus aureus* ATCC 25923	$3{,}4.10^9$	20
5	*Pseudomonas aeruginosa* ATCC 9027	$1{,}07.10^9$	9
6	*Candida albicans* ATCC 10231	4.10^8	11

Well diameter: 6 mm

* - inhibition zone with single cell colonies

CONCLUSIONS

The synthesis of 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) was described and investigated by UV-Vis, IR, FT-IR ATR and Raman spectroscopy. The preliminary results of our study showed that 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) could not serve as potential anticancer agent. Further investigations are needed to elucidate the exact mechanisms of this action and to exclude any cytotoxic effect on normal cells. The results for 3-methyl-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) showed that it has potential as antimicrobial agent against Gram positive bacteria. It presented moderate activity against yeast and low or no activity against the Gram negative bacteria included in the test.

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