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Scientific paper

Study on the Synthesis, Characterization and Bioactivities of 3-Methyl-9'-fluorenespiro-5-hydantoin

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Abstract

This work describes a method for synthesis, as well as *in vitro* antiproliferative and antibacterial investigation of 3-methyl-9'-fluorenespiro-5-hydantoin. The structure of the substituted fluorenylspirohydantoin derivative was verified by UV-Vis, FT-IR, Raman, ^{1}H NMR and ^{13}C NMR spectroscopy, and by using a combination of 2D NMR experiments, which included ^{1}H-^{1}H COSY, HMQC and HMBC sequences. The geometry of the compound was optimized by the B3LYP density functional with 6-31G(d) basis set and the ^{1}H and ^{13}C NMR spectra were predicted with the HF/6-31G(d) calculations at the optimized geometry. The anticancer activity of the 3-methyl-9'-fluorenespiro-5-hydantoin was determined in suspension cell lines originating from tumors in humans (WERI-Rb-1). The cytotoxic effect was evaluated by WST-assay (Roche Applied Science). The antimicrobial effect of the compound against Gram-negative, Gram-positive bacteria and the yeast *Candida albicans* was investigated.

Keywords: 3-methyl-9'-fluorenespiro-5-hydantoin; NMR spectra; cytotoxic activity; antimicrobial effect

1. Introduction

Hydantoins, or 2,4-imidazolidinediones are compounds of considerable interest both from a chemical and biological point of view.[1] Several compounds of this class have shown a pharmaceutically useful activity that led in some cases to clinical applications. In particular, 5-substituted and 5,5-disubstituted hydantoins are important medicinal compounds: phenytoin, or 5,5-diphenylhydantoin, is widely used as an anticonvulsant agent, for the treatment of epilepsy, and as a cardiac antiarrhytmic agents.[2,3] Among the medicinally useful properties exhibited by other 5-substituted hydantoins, at least their antidepressant and antiviral activities, the inhibition of platelet aggrega-

tion as well as human aldose reductase and human leukocyte elastase inhibition are worth mentioning. A number of other biological activities of hydantoin derivatives are known, including possible uses as herbicides,[4–7] fungicides[8,9] and insecticides.[10–15]

Lee *et al.* presented the molecular modeling of six structurally diverse ARIs (aldose reductase inhibitors), being carried out at the active site of aldose reductase to probe the charge interactions between the ionizable group (*e.g.* carboxylate or hydantoin) of the ARIs and the positively charged His 110.[16] An attempt was also made to correlate the binding mode of these structurally diverse inhibitors to observed inhibitory activity. Palm *et al.* investigated the influence of diabetes-induced changes in oxygen tension and consumption in relation to regional renal metabolism in rats.[17] In the second set of experiments, the putative role of the polyol pathway for hyperglycaemia-induced alterations in renal metabolism was studied. Sugiyama *et al.* reported a method for the *in vitro* isolation of a non-covalent complex formed in solution by the interaction of human muscle or rat lens aldose reductase with either $NADP^+$ or NADPH and the aldose reductase inhibitors tolrestat, AL1576 (2,7-difluorospirofluorene-9,5'-imidazolidine-2',4'-dione), or ponalrestat.[18] Kato *et al.* investigated the effects of novel aldose reductase inhibitors, M16209 (1-(3-bromobenzo[*b*]furan-2-ylsulfonyl)hydantoin) and M16287 (1-(3-chlorobenzo[*b*]furan-2-ylsulfonyl)hydantoin), on neuropathy in streptozotocin-induced (STZ) diabetic rats.[19] The effect of a single oral administration of M16209, a novel aldose reductase inhibitor, on serum glucose was investigated by Nakayama *et al*.[20] The group of Nakayama investigated the stimulatory effects of M16209 on insulin secretion using isolated, perfused pancreases in rats.[21] M16209 showed no appreciable effect on ATP-sensitive K^+-channels in pancreatic β-cells. Two potent aldose reductase inhibitors, l-[(2,5-dichlorophenyl)sulfonyl]hydantoin (Di-ClPSH) and 1-[β-naphthyl)sulfonyl]hydantoin (β-NSH), were tested for usefulness in the treatment of diabetic and galactosemic complications in animal experiments.[22]

Sorbitol formation from glucose, catalyzed by the enzyme aldose reductase, is believed to play a role in the development of certain chronic complications of diabetes mellitus. Spirohydantoins derived from five- and six-membered ketones fused to an aromatic ring or ring system inhibit aldose reductase isolated from calf lens. *In vivo* these compounds are potent inhibitors of sorbitol formation in sciatic nerves of streptozotocinized rats. Optimum *in vivo* activity is reached in spirohydantoins derived from 6-halogenated 2,3-dihydro-4*H*-l-benzopyran-4-ones (4-chromanones). In 2,4-dihydro-6-fluorospiro[4*H*-l-benzopyran-4,4'-imidazolidine]-2',5'-dione, the activity resides exclusively in the 4S isomer, compound 115 (CP-45,634, USAN: sorbinil). This compound is currently being used to test, in humans, the value of aldose reductase inhibitors in the therapy of diabetic complications.[23] A series of 27 hydantoins was prepared and tested as antitumor agents. These were variously substituted at the 5 position but with special emphasis on the substituents (chloro, acetyl, chloroacetyl, and methyl) at the 1 and/or 3 positions. The most active compound was 5,5-bis(4-chlorophenyl)-l,3-dichlorohydantoin with a T/C value of 190% against P-388 lymphocytic leukemia in mice.[24]

Hydantoinases are valuable enzymes for the production of optically pure D- and L-amino acids. They catalyze the reversible hydrolytic ring cleavage of hydantoin or 5-monosubstituted hydantoins and therefore are classified in the EC-nomenclature as cyclic amidases C 3.5.2 group.[25] Hydantoinases have been classified into D-, L-, unselective or ATP-requiring enzymes due to their substrate specificity, stereoselectivity and cofactor dependency. From recent findings based on protein sequence data all hydantoin cleaving enzymes, with the exception of the ATP-dependent *N*-methylhydantoinases, belong to a protein superfamily of »amidohydrolases related to urease«[26] and seem to have evolved from a common ancestor in a divergent evolution.[27] A D-specific hydantoinase has been purified to homogeneity from *Arthrobacter crystallopoietes* DSM 20117 with a yield of 5% related to the crude extract.[28] The group of Yamada was the first to study intensively the D-selective cleavage of 5-monosubstituted hydantoins in microorganisms.[29] They postulated the identity of microbial D-hydantoinases with dihydropyrimidinases and proved this hypothesis for the enzyme from *Pseudomonas striata*.[30] In the meantime, several publications described various similar D-selective microbial hydantoinases from microorganisms, such as *Pseudomonas fluorescens* DSM 84,[31] *Pseudomonas sp.* AJ11220,[32,33] *Agrobacterium sp.* IP-I 671,[34,35,40] several *Bacillus spp*.[36–38] and even from anaerobic microorganisms.[39] However, recently a hydantoinase from *Agrobacterium* was identified which exhibits no dihydropyrimididase activity.[34] DL-5-Monosubstituted hydantoins are converted to D-amino acids via *N*-carbamoyl-D-amino acids by some bacteria.[32,41,42] Takahashi *et al.*[30] revealed that in *Pseudomonas putida (P. striatu)* IFO 12996, D-hydantoinase is identical with dihydropyrimidinase, which catalyzes the cyclic ureide-hydrolyzing step of the reductive degradation of pyrimidine bases. The same results were obtained for other *Pseudomonas* species,[43,44] *Comamonas* species,[44] *Bacillus* species,[45] *Arthrobacter* species,[41] *Agrobacterium* species,[43] and rat liver.[46] Various 5-chloroarylidene-2-amino substituted derivatives of imidazoline-4-one were synthesized and evaluated for their activity *in vitro* against *Mycobacterium tuberculosis* and other type strains of bacteria and fungi. 2-Chloro- and 2,4-dichlorobenzylidene substituted hydantoins exhibited antimycobacterial effect.[47] The antimitotic effect of the investigated hydantoins was also examined. In the course of structure-activity relationship (SAR) studies and to explore the antiproliferative effect associated with the hydantoin framework, several diversely substituted diazaspiro hydantoins were synthesized.[48] Variation in the functional group at *N*-terminal of the hydan-

toin ring and coupling of different substituted aromatic acids in 4-aminocyclohexanone ring led to three sets of compounds. The antiproliferative effect of the compounds was evaluated *in vitro* using the MTT test against one normal cell line (NDF-103 skin fibroblast cells) and four human cancer cell lines (MCF-7 breast carcinoma cell line, HepG-2 hepatocellular carcinoma cell line, HeLa cervix carcinoma cell line and HT-29 colon carcinoma cell line) for the time period of 24 h. Among the series, some compounds exhibited interesting growth inhibitory effects against all four cell lines. The SAR studies revealed that the substitution at *N*-terminal in hydantoin ring played a key role in the antiproliferative activity.

Especially, it is important to note that the study of (9'-fluorene)-spiro-5-hydantoin/spiro-(fluorene-9,4'-imidazolidine)-2',5'-dione and its derivatives is mainly determined from the point of their biological activity. Such compounds are known as aldose reductase inhibitors[49–51] and some of them have antitumor activity.[52,53] In our previous works we reported several studies about different methods for synthesis of monothio and dithio- analogues of (9'-fluorene)-spiro-5-hydantoin.[54–56] Furthermore, a method for (4',5'-diaza-9'-fluorene)-spiro-5-hydantoin synthesis has been described.[57] Fluorenylspirohydantoins have a good ability to coordinate metal ions. Copper(II) and nickel(II) complexes of (9'-fluorene)-spiro-5-dithiohydantoin,[58] as well as platinum(II) complexes of (9'-fluorene)-spiro-5-hydantoin and (9'-fluorene)-spiro-5-(2-thiohydantoin)[59] have been described in this aspect. Moreover, we reported studies about synthesis, cytotoxicity and antibacterial activity of some fluorenylspirohydantoin derivatives[60–62] and their platinum(II) complexes.[59] Recently, we discussed the synthesis, characterization and quantum chemical investigation of new Pt(II) complexes of cyclohexanespiro-5-(2-thiohydantoin) and cycloheptanespiro-5-(2-thiohydantoin).[63] In our previous works we reported a method for synthesis, cytotoxicity and antibacterial activity of 3-amino-9'-fluorenespiro-5-hydantoin.[61] On the other hand, compounds containing fluorene ring have been proved as organic light emitting diodes (OLED) having application in the practice.[64] Although hydantoin compounds are investigated extensively, there are not many reports on their anticancer activity.

For this reason, the goal of the present paper is to describe a method for synthesis of 3-methyl-9'-fluorenespiro-5-hydantoin, its structural elucidation and biological properties (its cytotoxic and antimicrobial effects).

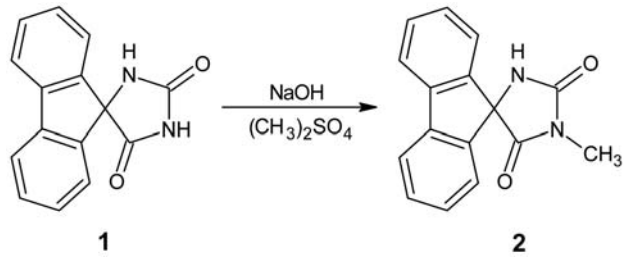
2. Experimental

2. 1. Instrumentation and Methods

All chemicals used were purchased from Merck and Sigma-Aldrich. UV/Vis 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'-fluorenespiro-5-hydantoin was obtained as 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. The Raman spectrum of the obtained product (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 400 cm^{-1} at resolution 2 cm^{-1} with 25 scans. The NMR spectra were taken on a Bruker Avance II+ 600 MHz 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.

2. 2. Synthesis of 3-Methyl-9'-fluorenespiro-5-hydantoin (2)

The initial 9'-fluorenespiro-5-hydantoin (**1**) (12.5 g, 0.05 mol) was dissolved in water solution of NaOH (2.5 g in 50 mL of water) and $(CH_3)_2SO_4$ (8 g, 0.063 mol) was added for 5 minutes at 40 °C (Scheme 1). The reaction mixture was stirred for 10 min at room temperature and was left overnight. The crystalline product **2** obtained was filtered off and recrystallized from methanol. Yield: 10.30 g (78%); m.p.: 227–228 °C; R_f = 0.59 (ethyl acetate : petroleum ether = 1 : 2).



1 **2**

Scheme 1. Synthesis of 3-methyl-9'-fluorenespiro-5-hydantoin (**2**)

The spectral data of **2**: UV (EtOH) λ_{max} = 306, 271, 235, 228, 210 nm. IR (KBr): ν 3461, 3232 (^{1}N–H), 3058–3041 (CH, arom.), 2946 (CH_3), 2814 (CH_3), 1971, 1936, 1775 (C^4=O), 1717(C^2=O), 1606, 1585, 1452 (CH_3), 1390 (CH_3), 1294, 1237, 1211, 1153, 1129, 1111, 1064 (C–O), 1042, 1033, 1020, 982, 951, 922, 883, 874, 785, 772, 756, 745, 713, 681, 658, 621, 603, 509, 499, 460, 422, 402 cm^{-1}. Raman: ν 3058 (CH, arom.), 3003 (CH, arom.), 2949 (CH_3), 2588, 1771 (C^4=O), 1607, 1585, 1490, 1454 (CH_3), 1351, 1298, 1234, 1180, 1159, 1128, 1021, 981, 886, 787, 756, 715, 683, 622, 566, 515, 422 cm^{-1}.

2. 3. WST-1 Cell Proliferation Assay

The cytotoxic effect of compound **2** was assessed on a suspension cell line using WST-1 assay (Cat. No11

644 807 001, Roche). The suspension retinoblastoma cells (WERI-Rb1, ATCC-HTB-169) were cultured in RPMI 1640 medium, containing 10% FCS, 100 μg/mL streptomycin and 100 units/mL penicillin. Cells were cultured at 37 °C 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 flat-bottom plates at a density of 6.5×10^4 cells/well (WERI-Rb1). After a cultivation period of 24 h, the compound was added at a concentration 100 μM on WERI-Rb1 cells and incubated for 24, 48 and 72 h, respectively. WST-1 was added to the cells at these time points and incubated for 4 h. After the 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.

2. 4. Antimicrobial Assay

The antimicrobial activity of **2** against Gram-positive bacteria – *Staphylococcus aureus* ATCC 25923 and *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 investigated using the agar diffusion method. Melted PCA (Scharlau) nutrient medium was inoculated through the addition of 1 mL of microbial suspension (1×10^{10} CFU/mL for the bacteria and 1×10^9 CFU/mL for the yeast) and was poured in Petri dishes, 20 mL in each dish. Wells with 7 mm diameter were made in the solidified and cooled agar medium. 50 μL of the tested substance solution (5.38 mg/mL in 15% DMSO) was pipetted into the wells. The current concentrations of the test microorganisms in the suspensions were as follow: *S. aureus* 3.4×10^9; *Bacillus subtilis* 2.21×10^9; *E.coli* 1.02×10^9; *S. enterica subsp enterica* 1.12×10^9; *P. aeruginosa* 1.07×10^9; *C. albicans* 4×10^9 cfu/mL. The Petri dishes were incubated at 37 °C for 24–48 h. The inhibition zone was measured. Zones with diameter more than 7 mm were considered zones of inhibition.

2. 5. Computational Details

To additionally verify the proposed assignments, quantum chemistry calculations were performed by using the Gaussian 98, Revision A.7.[65] For the geometry optimization the B3LYP density functional with 6-31G(d) basis set was used and for the ^{1}H and ^{13}C NMR spectra prediction the HF/6-31G(d) calculations were carried out at the optimized geometry.

3. Results and Discussion

A synthesis procedure for 9'-fluorenespiro-5-hydantoin methylation with diazomethane has already been described.[66] Here we present a new method for 3-methyl-9'-fluorenespiro-5-hydantoin (**2**) preparation. The method discussed here is based on the reaction of 9'-fluorenespiro-5-hydantoin with dimethyl sulfate. The target product was obtained with high yield (78%) and showed m.p. 227–228 °C. The synthesis of **2** was carried as shown in Scheme 1. The structure of **2** was determined by UV-Vis, FT-IR, Raman, ^{1}H NMR and ^{13}C NMR spectroscopy. Maxima in the UV/Vis spectrum of the **2** were observed at 306, 271, 235, 228, 210 nm. The IR band at 3232 cm^{-1} of **2** that was observed may refer to the stretching vibration of the N–H group of the hydantoin ring. The vibrational (N^1–H) stretching mode did not appear in the Raman spectrum. In the IR spectrum of the **2** the bands at 1775 cm^{-1} and 1717 cm^{-1} can be attributed to stretching vibrations of the two C=O groups of the hydantoin ring. In the Raman spectrum of **2** the one of the two C=O groups appeared at 1771 cm^{-1}. The other vibrational (C=O) stretching mode did not appear in the Raman spectrum. Several bands in the IR spectrum (3058, 3041 cm^{-1}) and in the Raman spectrum (3058, 3003 cm^{-1}) were for stretching vibrations of CH in fluorene moiety. In the IR spectrum of the **2** the bands at 2946 cm^{-1} and 2814 cm^{-1} can be attributed to stretching vibrations of the CH_3 group. In Raman spectrum of **2** the former vibration appeared at 2949 cm^{-1}.

The ^{1}H-broadband-decoupled ^{13}C NMR spectrum of **2** showed 10 signals: 6 pairs of atoms were magnetically equivalent. The two signals with the highest chemical shift in ^{13}C NMR spectrum, 173.06 and 157.58 ppm, were for the carbonyl groups (C^4=O) and (C^2=O). The signals at 71.54 and 25.49 ppm were for the spiro-carbon and methyl group. The structure of multiplets and coupling constants in ^{1}H NMR spectrum were consistent with the structure of **2**. The assignment of signal at 71.54 to the spiro carbon, C-9', was supported also by an HMBC correlation of HN with it (δ_H 8.87–δ_C 71.54). There was also an HMBC correlation δ_H 7.47–δ_C 71.54 which points out that this δ_H is for H-1'/8'. This inference and the COSY correlations allow to unambiguously assign all proton signals. As only the meta (vicinal) coupling ($^3J_{CH}$) in benzene rings is usually resolved,[67] the assignments of the quaternary carbons, C-1a', C-4a', C-5a' and C-8a', can be made (Table 1).

The effect of the compound **2** on the proliferation of WERI-Rb1 cells after 24 and 72 h of treatment is presented in Fig. 1. The results from the cytotoxicity assay on the human WERI-Rb-1 cell line showed that the product **2** reduced the number of tumor cells by around 2% after 24 h. It showed a significant cytotoxic effect after 72 h of treatment when cell vitality decreased by 80%.

The results for the antimicrobial activity of **2** are presented in Table 2. Compound **2** showed strong antimi-

Table 1. ^{1}H and ^{13}C NMR spectral data and ^{1}H-^{1}H COSY and HMBC correlations for **2** (600.13 MHz (^{1}H) and 150.903 MHz (^{13}C))[a,b]

Atom	δ (^{13}C) ppm	DEPT[b]	δ (^{1}H) ppm	Multiplicity (*J*, Hz)	^{1}H-^{1}H COSY[c]	HMBC[c]
1 (NH)	–	–	8.87	s		2, 4, 9’
2 (C=O)	157.58	C	–	–	–	–
4 (C=O)	173.06	C	–	–	–	–
1’ / 8’	124.29	CH	7.47	d (7.9)	2’	3’, 4a’, 9’, 2’[d]
1a’ / 8a’	143.13	C	–	–	–	–
2’ / 7’	128.76	CH	7.35	td (7.5, 0.9)	1’, 3’	1a’, 3’[d], 4’, 9’[d]
3’ / 6’	130.36	CH	7.50	dd (7.5, 0.9)	2’, 4’	1’, 2’[d], 4a’
4’ / 5’	121.20	CH	7.90	dt (7.5, n/a)	3’	1a’, 2’, 5a’[d]
4a’ / 5a’	141.16	C	–	–	–	–
5 (9’)	71.54	C	–	–	–	–
	25.49	CH_3	3.00	s		2, 4

[a] In DMSO-d_6 solution. All these assignments were in agreement with COSY, HMQC and HMBC spectra. [b] Abbreviations: DEPT, Distortionless Enhancement by Polarization Transfer spectrum; ^{1}H-^{1}H COSY – proton-proton homonuclear correlation spectrum; HMQC, Heteronuclear Multiple Quantum Correlation experiment; HMBC, Long range ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation experiment. [c] For brevity these correlations are given only in one of the benzene rings. [d] These correlations are weak.

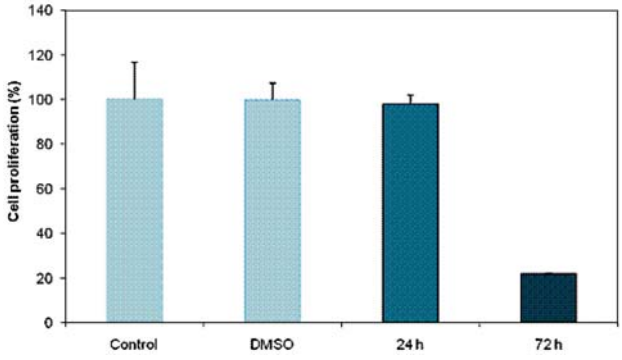


Fig. 1. Effect of the compound **2** (100 μM) on the proliferation of WERI-Rb1 after 24 h and 72 h of treatment

crobial effect only against *Bacillus subtilis* (inhibition zone 15 mm) and moderate antimicrobial activity against *S. aureus* and *P. aeruginosa* (inhibition zones 9 mm). 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.

4. Conclusions

The method for synthesis of 3-methyl-9’-fluorenespiro-5-hydantoin (**2**) was presented. The structure of the obtained product **2** was determined by UV-Vis, FT-IR, ^{1}H, ^{13}C NMR and Raman spectroscopy, as well as by means of one- and two-dimensional NMR techniques, including HMQC, ^{1}H–^{1}H COSY, and HMBC spectra. The preliminary results of our study showed that the compound could serve as a 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 the compound **2** showed

Table 2. Antimicrobial activity of 3-methyl-9’-fluorenespiro-5-hydantoin (**2**)

Run	Test microorganism	Viable cells count in the nutrient medium, cfu/mL	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}	15/15[a]
4	*Staphylococcus aureus* ATCC 25923	3.4×10^{9}	9/9
5	*Pseudomonas aeruginosa* ATCC 9027	1.07×10^{9}	9/9
6	*Candida albicans* ATCC 10231	4×10^{8}	–

Well diameter: 6 mm
[a] inhibition zone with single cell colonies.

that it has potential as antimicrobial agent against Gram positive bacteria.

The numbered structure of **2**, complete spectral data, with enlarged detailed sections for multiplets and cross peaks in NMR spectra, as well as the archive Gaussian job results are included in Supporting Information.

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Povzetek

V tem delu predstavljamo sintezo ter *in vitro* študijo antiproliferativnega in antibakterijskega delovanja 3-metil-9'-fluorenspiro-5-hidantoina. Strukturo substituiranega fluorenilspirohidantoinskega derivata smo potrdili z UV-Vis, FT-IR, Ramansko, ^{1}H NMR in ^{13}C NMR spektroskopijo kot tudi s kombinacijo 2D NMR eksperimentov, ki so vključevali ^{1}H-^{1}H COSY, HMQC in HMBC sekvence. Geometrijo spojine smo računsko optimizirali z B3LYP gostotnostnim funkcionalom z baznim setom 6-31G(d) ter simulirali ^{1}H in ^{13}C NMR spektre s HF/6-31G(d) izračuni za optimizirano geometrijo. Delovanje proti raku 3-metil-9'-fluorenspiro-5-hidantoina smo določili v suspenziji celičnih linij, ki so izvirale iz človeških tumorjev (WERI-Rb-1). Citotoksičnost smo določili z metodo WST (Roche Applied Science). Raziskali smo tudi antimikrobni učinek spojine proti Gram-negativnim in Gram-pozitivnim bakterijam ter proti kvasovkam *Candida albicans*.

Contents

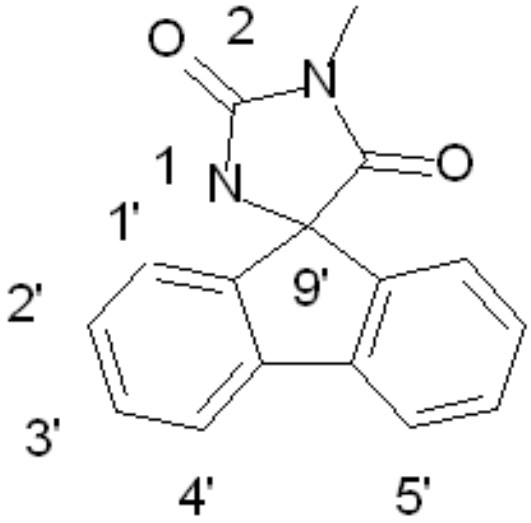

Figure 1a. Structure of 3-methyl-9′-fluorenespiro-5-hydantoin, **2**. The numbering of the atoms is only for spectral assignments. The numbering 9’ is also 5.

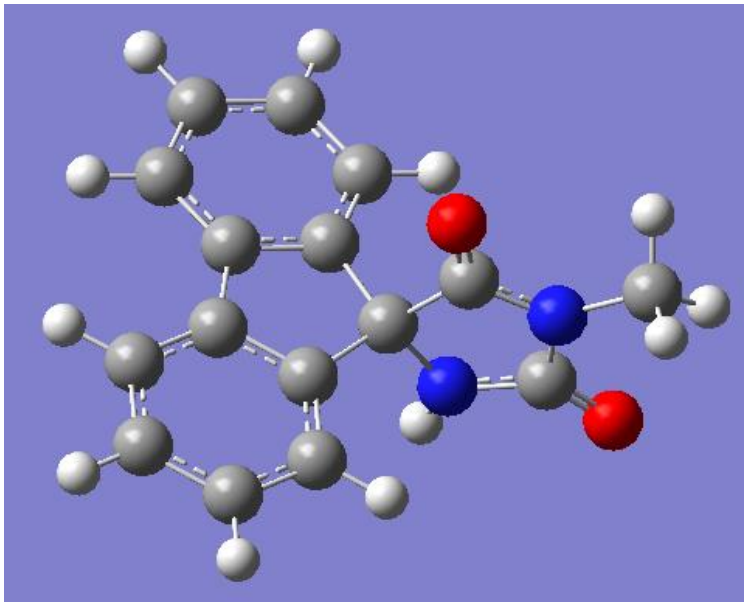

Figure 1b. The computed structure of 3-methyl-9′-fluorenespiro-5-hydantoin, **2**; geometry optimization by the B3LYP density functional with 6-31G(d) basis set.

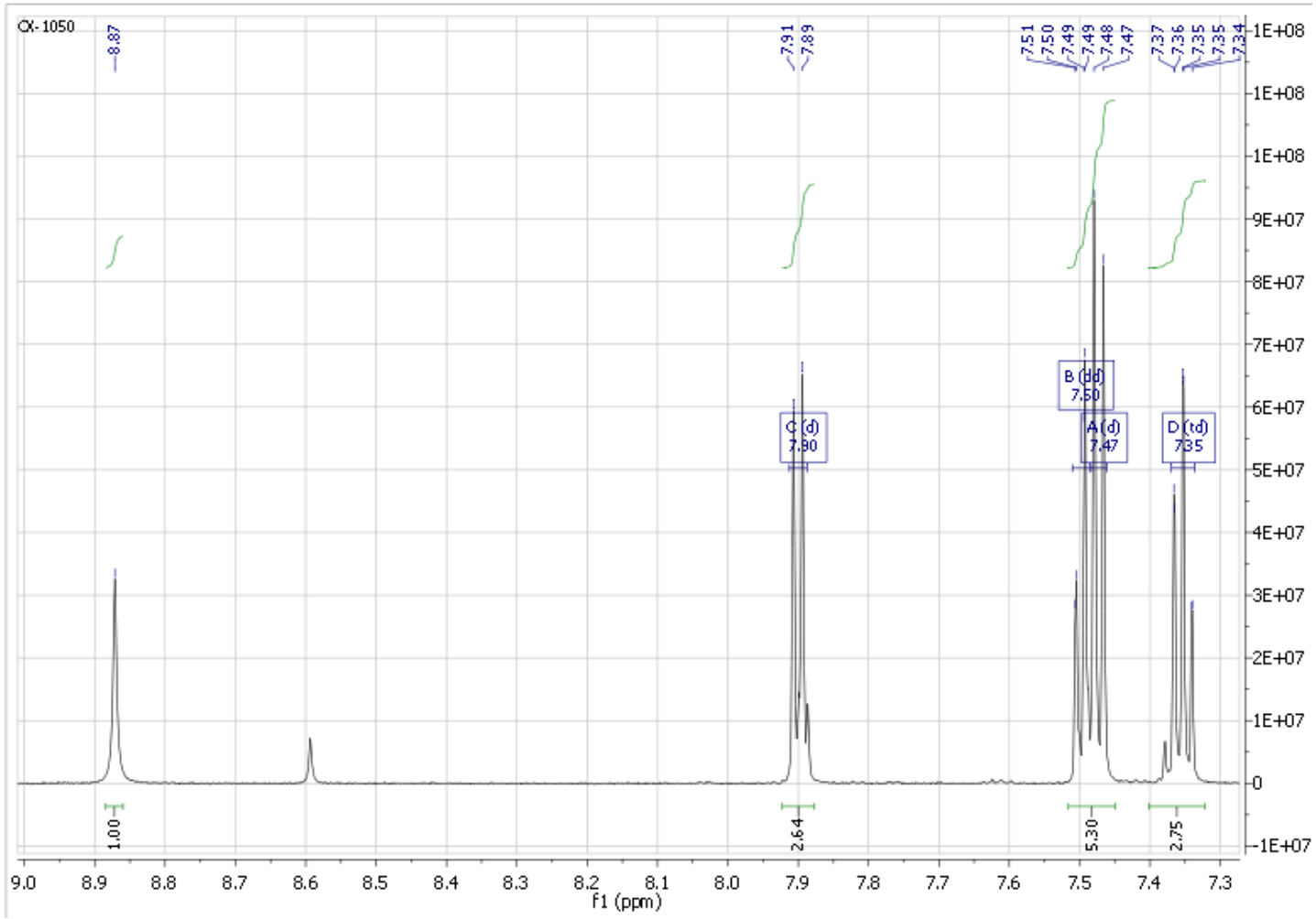

Figure 2. Part of the ^{1}H NMR spectrum of **2.**

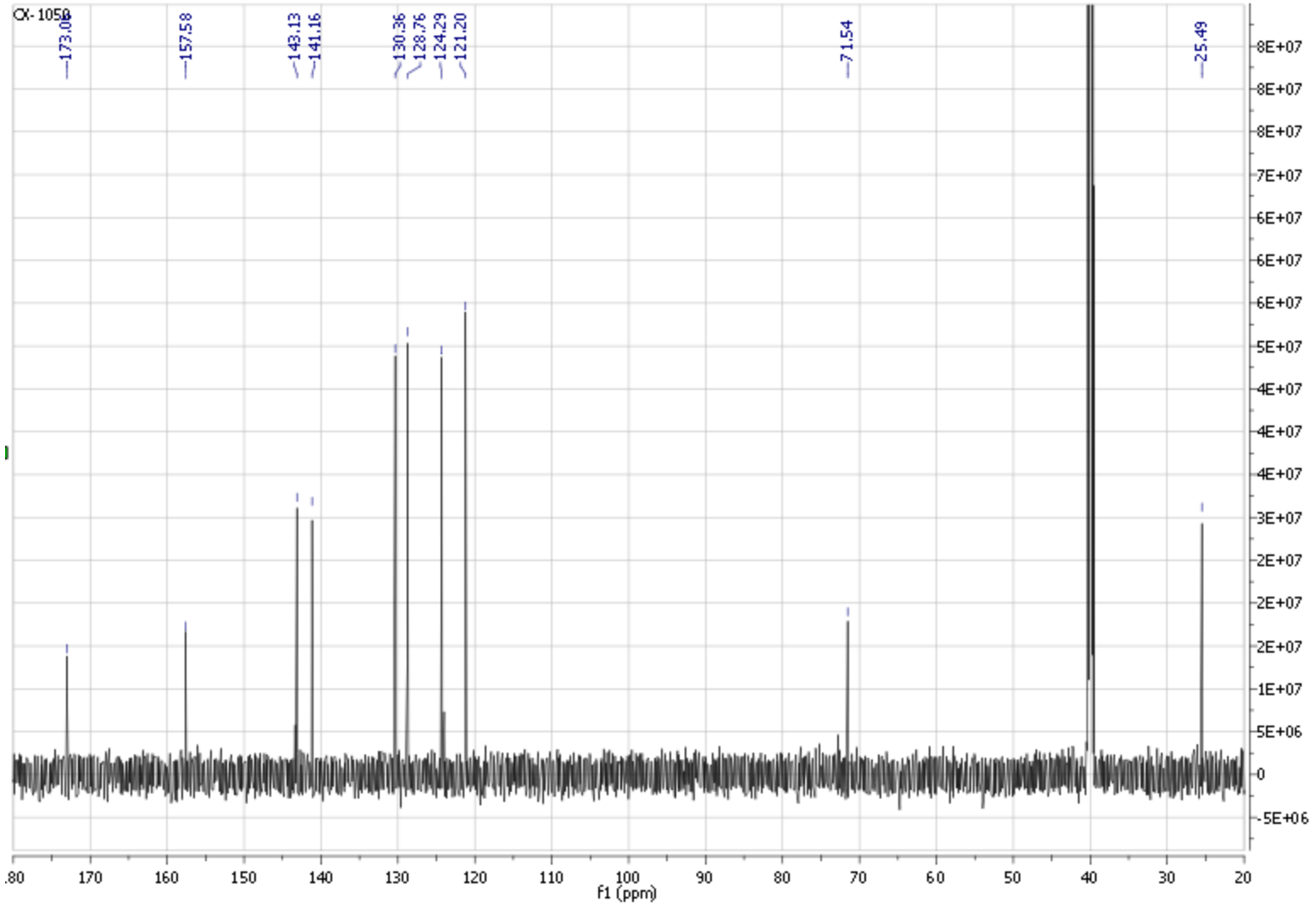

Figure 3. The ^{1}H-broadband-decoupled ^{13}C NMR spectrum of **2**. The signals at 173.06, 157.58, 143.13, 141.16 and 71.54 ppm are missing in DEPT-135 experiment.

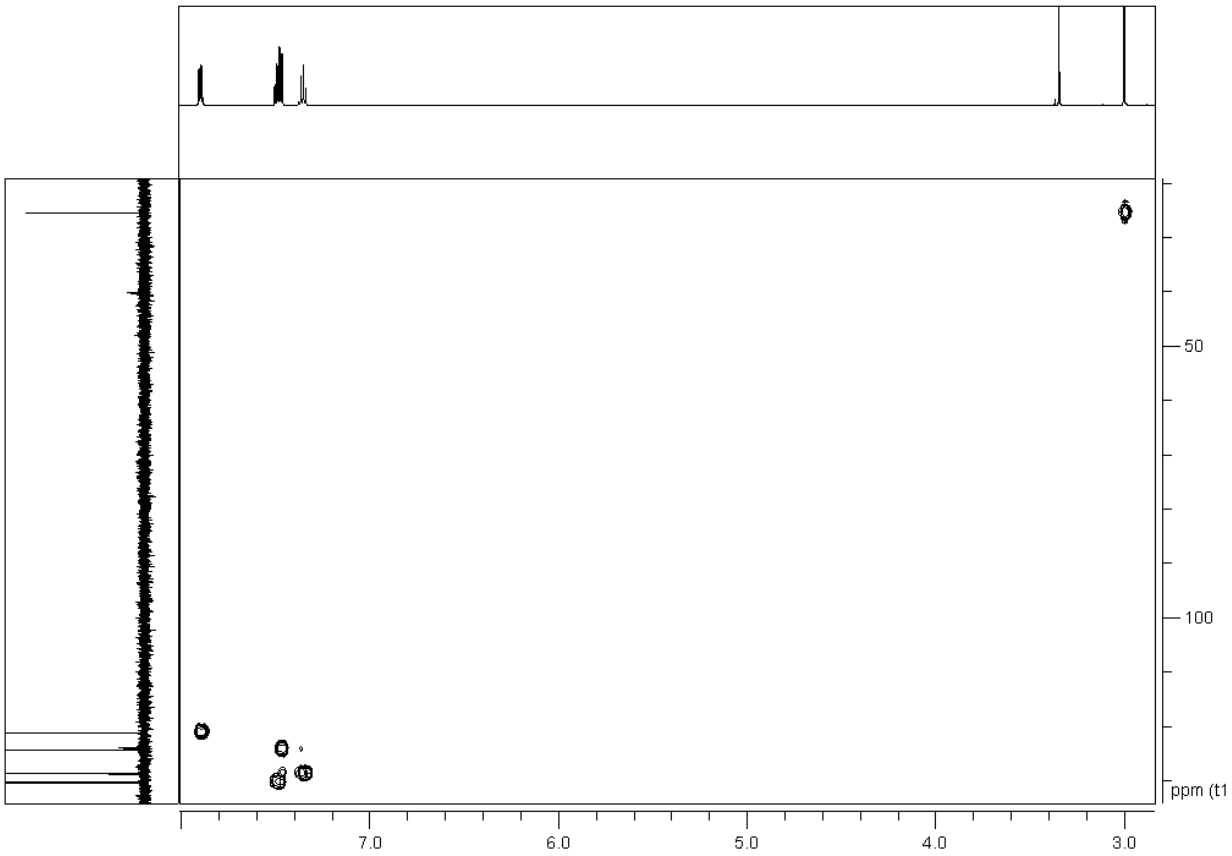

Figure 4. The HMQC spectrum of **2**. 1D trace (left) is ^{13}C DEPT-135 NMR spectrum and 1D trace (top) is ^{1}H NMR spectrum.

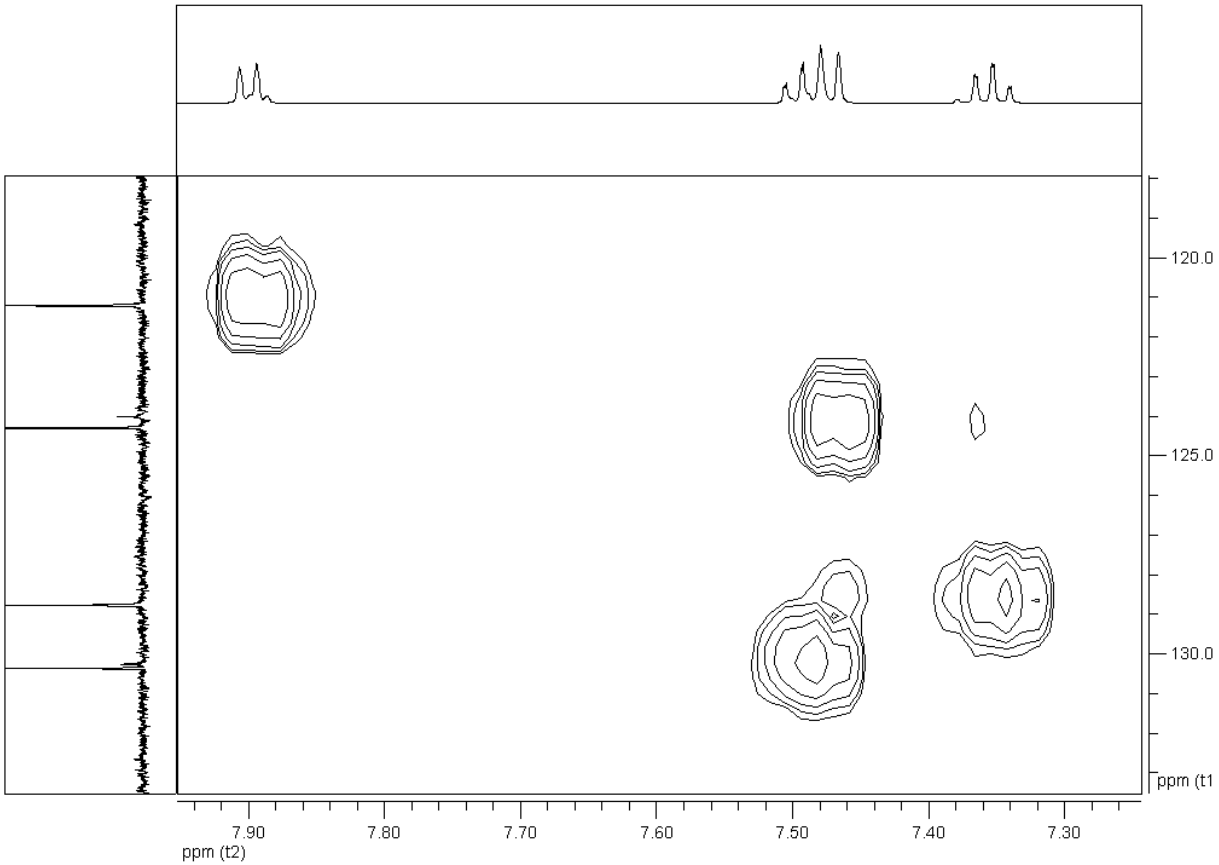

Figure 5. The detail of HMQC spectrum of **2**. 1D trace (left) is ^{13}C DEPT-135 NMR spectrum and 1D trace (top) is ^{1}H NMR spectrum.

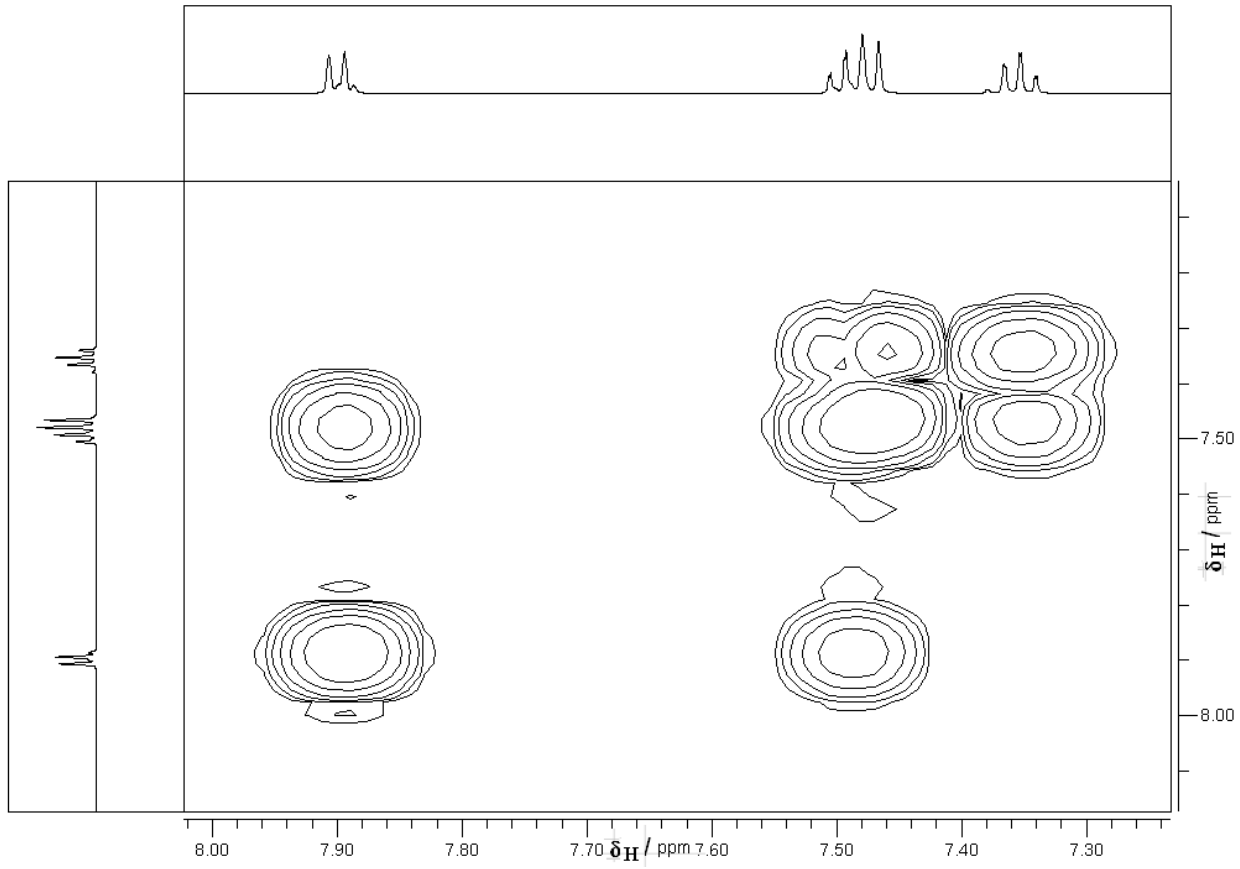

Figure 6. The detail of COSY spectrum of **2**. Both 1D traces (top and left) are ^{1}H NMR spectrum of the compound.

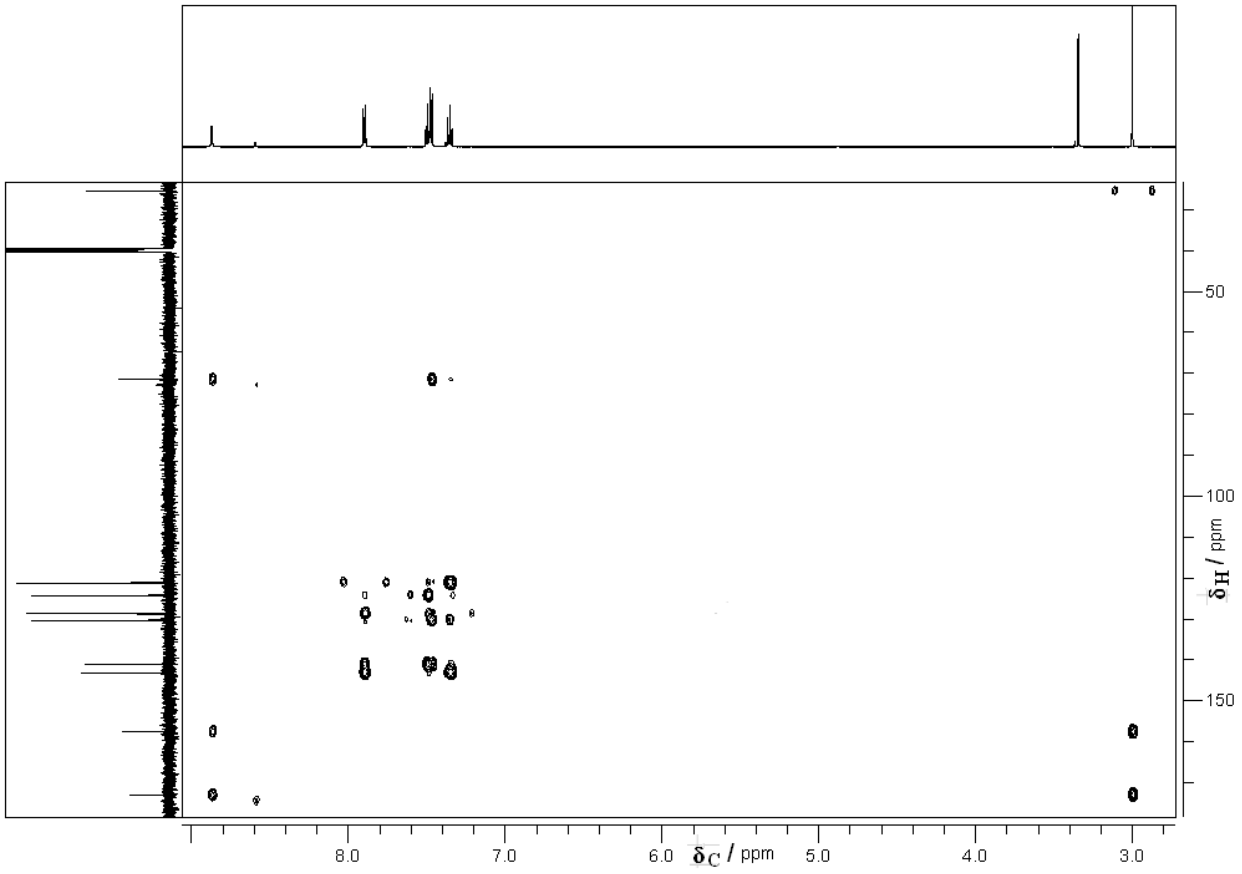

Figure 7. The HMBC spectrum of 2. 1D trace (left) is ^{13}C NMR spectrum and 1D trace (top) is ^{1}H NMR spectrum.

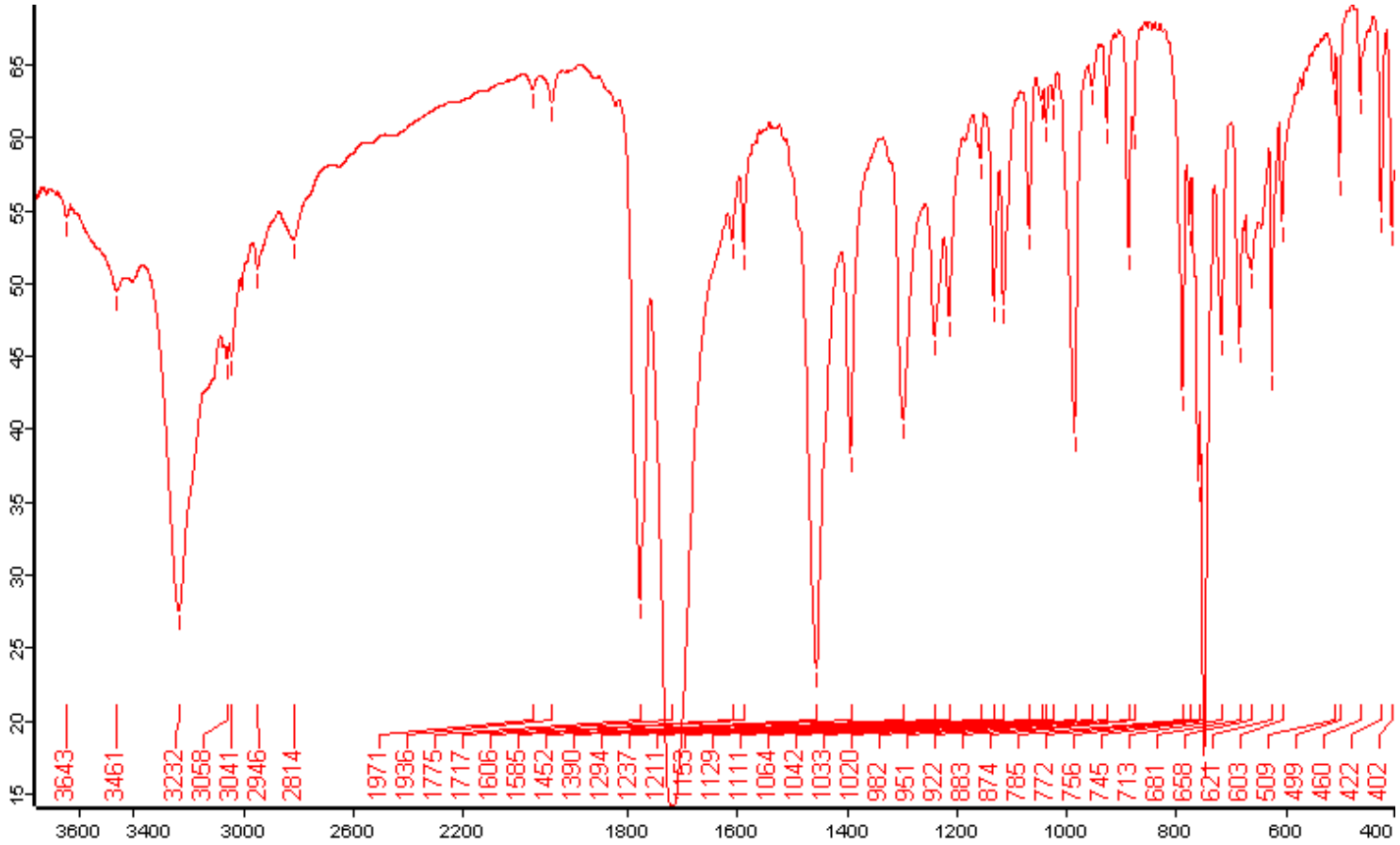

Figure 8. The IR spectrum of **2**.

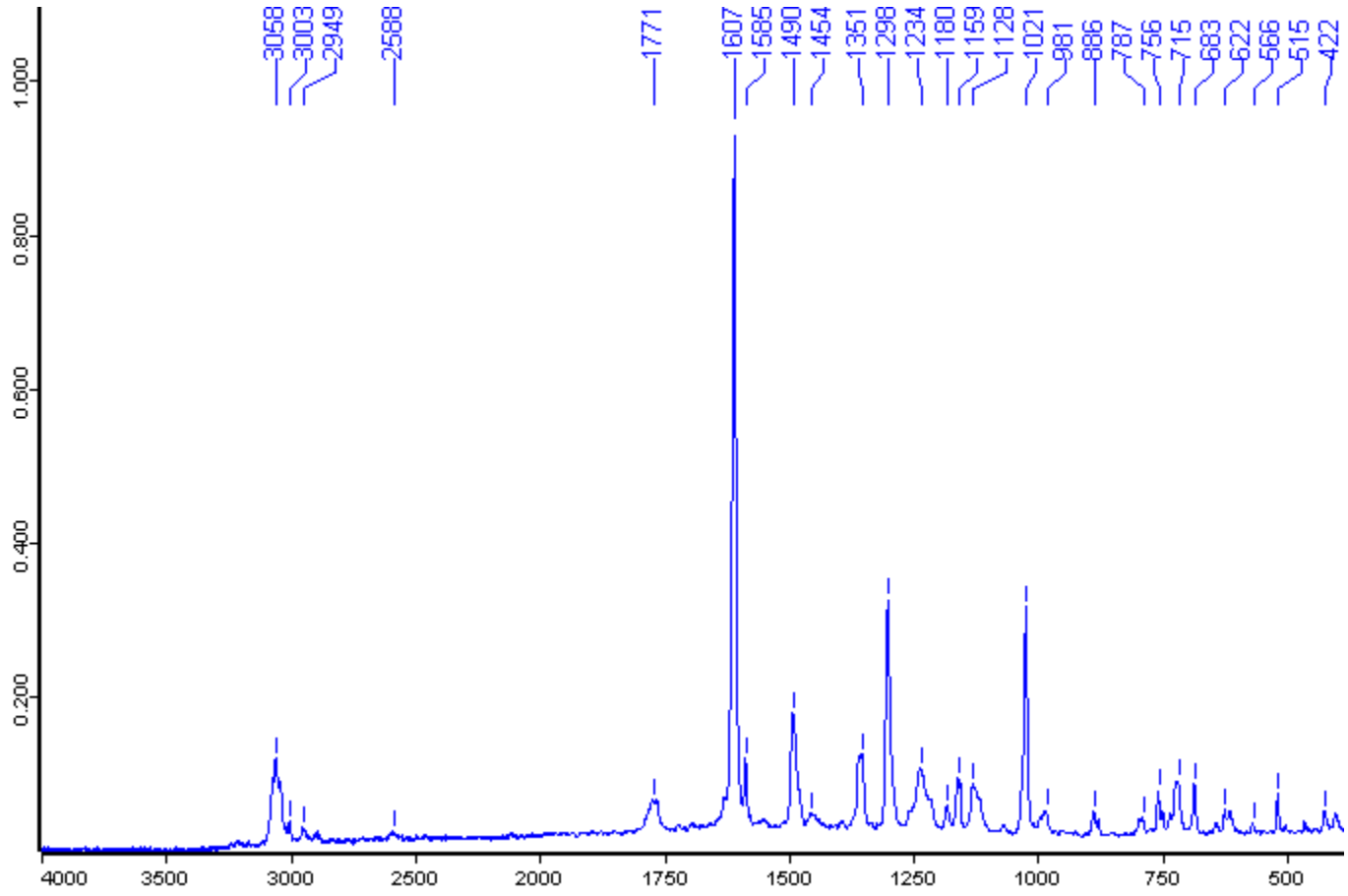

Figure 9. The Raman spectrum of **2**.

Table 1. Experimental and **calculated** ^{1}H and ^{13}C NMR spectral data for **2**

Atom	$\delta(^{13}C)$ ppm	DEPT [b]	$\delta(^{1}H)$ ppm	**$\delta(^{13}C)$, calc.** ppm	**$\delta(^{1}H)$, calc.** ppm
1 (NH)	–	–	8.87	**–**	**3.75**
2 (C=O)	157.58	C	–	**154.53**	**–**
4 (C=O)	173.06	C	–	**172.14**	**–**
1’ / 8’	124.29	CH	7.47	**124.80**	**7.58**
1a’ / 8a’	143.13	C	–	**140.98**	**–**
2’ / 7’	128.76	CH	7.35	**126.75**	**7.50**
3’ / 6’	130.36	CH	7.50	**129.89**	**7.73**
4’ / 5’	121.20	CH	7.90	**120.52**	**8.00**
4a’ / 5a’	141.16	C	–	**140.87**	**–**
5 (9’)	71.54	C	–	**64.18**	**–**
	25.49	CH_3	3.00	**24.41**	**3.13**

The archive Gaussian job results (for the structure optimization)

N-N= 1.491542429962D+03 E-N=-5.021454023800D+03 KE= 8.687508062063D+02
1|1|UNPC-UNK|FOpt|RB3LYP|6-31G(d)|C16H12N2O2|PCUSER|21-Jun-2013|0||# O
PT B3LYP/6-31G(D) GEOM=CONNECTIVITY||CX1050 (Petya) / # opt b3lyp/6-31
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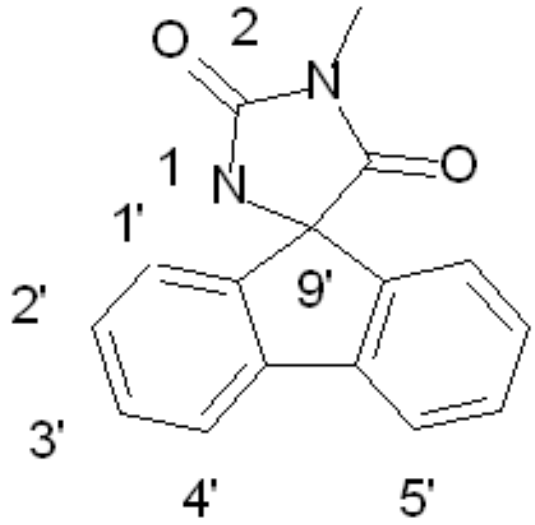

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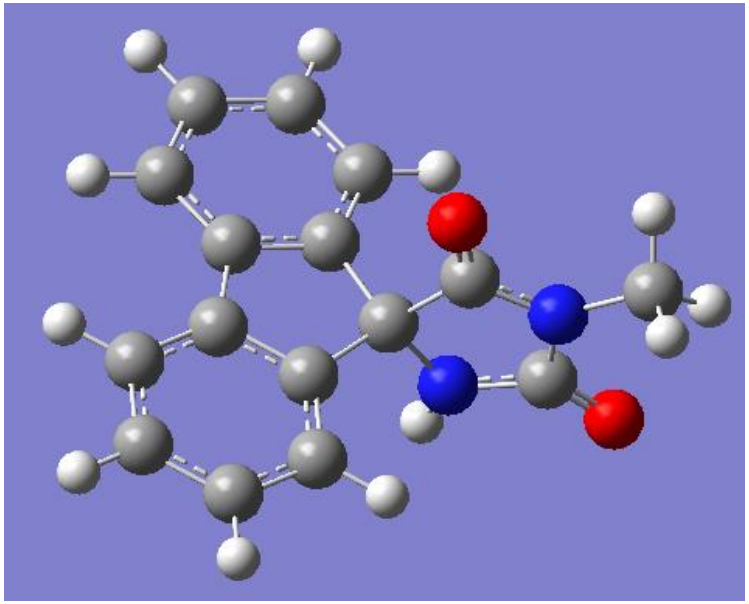

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