New N-acylhydrazones with Potential Cytotoxic Activity

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*Some new N-acylhydrazones **7a-c** having dibenzo[a,d][7]annulene moiety were synthesized by condensation of corresponding hydrazide **5** with aromatic aldehydes. The structures of these newly compounds were elucidated by IR, ^{1}H-NMR, ^{13}C-NMR, and elemental analysis. In the view of the therapeutical potential of the newly compounds, we evaluated their toxicological profile using Daphnia magna bioassay.*

Keywords: acylhydrazone, 5H-dibenzo[a,d][7]annulene, cytotoxicity, Daphnia magna bioassays

Malignant neoplasms along with circulatory system diseases represent one of the most serious threats against human health in the world. Overall, cancer is the second leading cause of death following heart disease [1].

Given the large social incidence and mortality rate which is continuously increasing, the chances of limiting the tumor development and of extending life using the present synthetic cytostatic drugs are still uncertain. In this context, the therapeutics introduction of more effective antitumor drugs, which may selectively inhibit the growth of tumor cells, but do not excessively affect normal cells and do not disturb the normal cellular processes, is a challenge for the organic synthesis chemistry involved in a hot field, that of improving the quality of life.

The hydrazones has been intensively studied in recent years.The hydrazones and their acylated derivatives are known for a wide range of pharmacological activities, such as analgesic, vasodilatory, antiviral, antioxidant, antidepressant, antitubercular, intestinal antiseptic, anti-inflammatory, antimicrobial, anticonvulsant [2-11].

Recent researches have shown antitumor properties of hydrazone derivatives, which are also due to their antiproliferative action: hydrazones derived from Plumbagin acting against the tumor on certain cell lines involved in breast cancer [12], hydrazones derivatives acting against the tumor on cell lines involved in ovarian cancer and leukemia [13], lung cancer, A-549 [14], prostate cancer [15], and brain tumors [16].

Considering these aspects, in the present work, we designed a set of new N-acylhydrazones with dibenzo[a,d][7]annulene fragment with potential cytotoxic activity, using rational drug design approach. The Lipinski law (Ro5) was used to assess if the compounds obtained possess properties that would make it possible for oral administration. Selected structures were further analyzed using *in silico* toxicity prediction software to estimate the LC50 on *Daphnia magna*. Based on the findings, we synthesized the new N-acylhydrazones starting from 2-(5H-dibenzo[a,d][7]annulen-5-yl)acetohydrazide. The newly synthesized compounds were characterized by IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis. In the view of the therapeutical potential of the newly compounds, we evaluated their toxicological profile using *Daphnia magna* bioassay.

Experimental part

All reagents were purchased from the Merck, Sigma-Aldrich and Fluka Companies. Melting points were determined on a Boetius apparatus and were uncorrected. The IR spectra were recorded in KBr pellets on a Vertex 70 Bruker spectrometer. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Gemini 300BB spectrometer (300 MHz for H and 75 MHMHz for C), using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. The content of C, H, and N was assayed using an ECS-40-10-Costeh microdosimeter.

Biological determinations were performed under constant temperature and light conditions using a Sanyo MLR-351 H, USA climatic chamber (25 ± 1 °C, a 16 h photoperiod and 8 h of darkness).

The key intermediate, 2-(5H-dibenzo[a,d][7]annulen-5-yl)acetohydrazide **5,** was prepared in several steps according to the literature method, starting from the dibenzosuberenone **1** (Scheme 1). The new N-acylhydrazones **7a-c** were synthesized by heating the 2-(5H-dibenzo[a,d][7]annulen-5-yl)acetohydrazide with aromatic aldehydes, in solvent ethanol [11,17].

General procedure for the preparation of 2-(5H-dibenzo[a,d][7]annulen-5-yl)-N'-[(R)methylidene] acetohydrazide

To a solution of 2-(5*H*-dibenzo[a,d][7]annulen-5-yl)acetohydrazide **5** (0.004 mol) in absolute ethanol (30-50mL) 0.004mol of the corresponding aromatic aldehyde **6a-c** was added. The mixture was refluxed for 6-12 h (Scheme 1). On cooling the reaction content to room temperature, a solid appeared. This was filtered off and recrystallized from ethanol to obtain the N-acylhydrazone.

2-(5H-dibenzo[a,d][7]annulen-5-yl)-N'-[(2-hydroxy)phenylmethylidene]-acetohydrazide (7a): Yield: 87%; *m.p.* 235-236C°; elemental analysis: anal. calcd. for $C_{24}H_{20}N_2O_2$ (368 g/mol): C, 78.24; H, 5.47; N 7.60; found: C, 78.24; H, 5.46; N 7.62; UV-Vis (CH_3OH) (λ max): 233.7, 290.2; IR (KBr, cm^{-1}): 3457 m, 3181 m, 3069 m, 3020 m,

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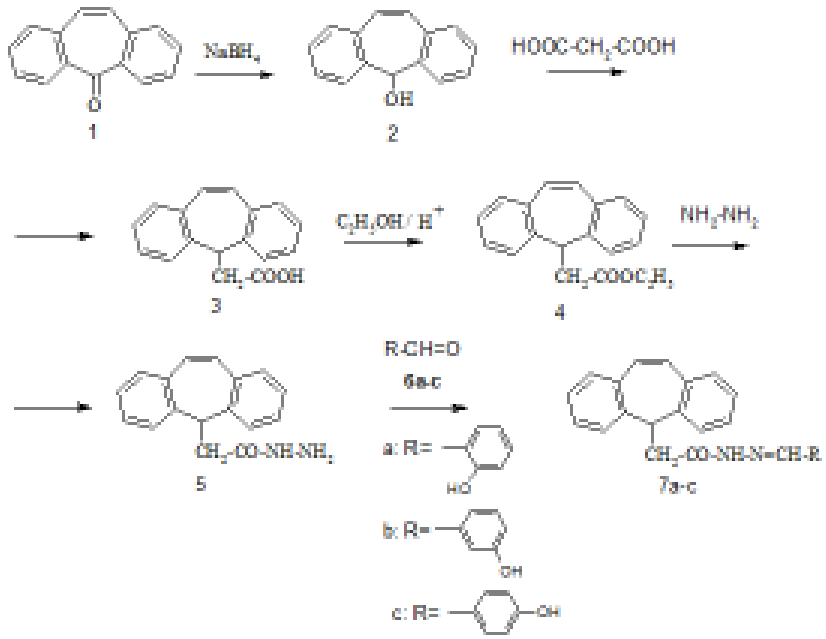


Scheme 1. Reaction pathway to the target compound **7a-c**

2978 m, 2888 m, 1663 s, 1622 m, 1606 s, 1574 w; ^{1}H-NMR (300 MHz, DMSO-d6, δ, ppm, *J*, Hz): 12.01 (s, NH, eq); 11.38 (s, NH,ax); 11.33 (s, NH,eq); 11.06 (s, NH,ax); 8.42(s, OH,eq); 8.31 (s, OH,ax); 8.18 (s, CH =N, ax); 8.11 (s, CH=N, eq); 6.80-7.60 (m,12H, Harom); 7.05 (2H, s, H$^{10'-11'}$, ax); 7.03 (2H, s, H$^{10'-11'}$, eq); 4.62 (H$^{5'}$, t, 7.6, ax); 3.80 (H$^{5'}$, t, 7.6, eq); 2.97 (2H, H$^{12'}$, d, 7.6, eq); 2.60 (2H, H$^{12'}$, d, 7.6, ax); ^{13}C-NMR (75 MHz, DMSO-d6, λ, ppm): 172.08 (C=O, eq); 171.74 (C=O, ax); 166.29 (Cq, ax), 157.23 (Cq, ax), 156.50 (Cq, eq); 156.36 (Cq, eq); 146.49 (CH=N, eq); 143.33 (CH=N, eq); 140.68 (CH=N, eq); 139.79 (CH=N, eq); 139.56 (Cq, ax); 139.35 (Cq, eq); 131.35 (CH, eq); 131.26(CH, eq); 130.99 (CH, eq); 130.84 (CH, ax); 129.72 (CH, ax); 129.65 (CH, ax); 129.59 (CH, ax); 129.37 (CH, eq); 128.92 (CH, ax); 128.81 (CH, eq); 128.58 (CH, eq); 127.71 (CH, eq); 126.67 (CH, ax); 126.67 (CH, eq); 125.62 (CH, eq); 122.95 (CH, eq); 120.38 (Cq, eq); 120.06 (Cq, eq); 118.62 (Cq, eq); 118.57 (Cq, ax); 49.15 (CH, 55.55 eq); 49.10 (CH, ax); 35.00(CH$_2$, ax); 33.88 (CH$_2$, eq); 32.84 (CH$_2$, eq);

2-(5H-dibenzo[a,d][7]annulen-5-yl)-N'-[(3-hydroxy)phenylmethylidene]-acetohydrazide (7a): Yield: 81%; *m.p.* 156-158C°; elemental analysis: anal. calcd. for $C_{24}H_{20}N_2O_2$ (368 g/mol): C, 78.24; H, 5.47; N 7.60; found: C, 78.25; H, 5.47; N, 7.58; UV-Vis (CH_3OH) (λ max): 224.3, 289.7; IR (KBr, cm^{-1}): 3354 m, 3262 m,3181 m, 3065 m, 3018 m, 2969 m, 2888 m, 1640 s), 1620 s, 1585m, 1536 w, 1491m; ^{1}H-NMR (300 MHz, DMSO-d6, δ, ppm, *J*, Hz): 11.73 (s, NH, eq); 11.32 (s, NH, eq); 11.08 (s, NH, ax); 10.97 (s, NH, ax); 8.12 (s, CH=N, eq); 8.00 (s, CH=N, ax); 7.89 (s, CH=N, eq); 7.72 (s, CH=N, ax); 6.70-7.40 (m,12H, Harom); 7.05 (2H, s, H$^{10'-11'}$, ax); 7.04 (2H, s, H$^{10'-11'}$, eq); 4.68 (H$^{5'}$, t, 7.6, ax); 3.75 (H$^{5'}$, t, 7.6, eq); 2.98 (2H, H$^{12'}$, d, 7.6, eq); 2.58 (2H, H$^{12'}$, d, 7.6, ax); ^{13}C-NMR (75 MHz, DMSO-d6, λδ, ppm): 172.43 (C=O, eq); 172.14 (C=O, ax); 166.90 (Cq); 157.77 (Cq); 157.68 (Cq-O); 146.21 (CH=N, eq); 145.96 (CH=N, eq); 143.45 (CH=N, eq); 142.71 (CH=N, ax); 139.83 (Cq, ax); 139.75 (Cq, ax); 139.54 (Cq, eq); 135.62 (Cq); 135.55 (Cq); 134.97 (Cq); 135.62 (Cq, ax); 133.89 (Cq); 131.36 (CH, eq); 130.86 (CH, ax); 129.95 (CH, eq); 129.86 (CH, eq); 129.72 (CH, ax); 129.65 (CH, ax); 129.59 (CH, ax); 129.74 (CH); 129.65 (CH); 129.58 (CH, ax); 128.90 (CH); 128.56 (CH, eq); 127.70 (CH); 126.65 (CH, ax); 126.60 (CH, ax); 125.65 (CH, eq); 125.58 (CH, eq); 49.42 (CH, 55.55 ax); 49.42 (CH, ax); 49.42 (CH, eq); 35.32 (CH$_2$, ax); 32.92 (CH$_2$, eq);

2-(5H-dibenzo[a,d][7]annulen-5-yl)-N'-[(4-hydroxy)phenylmethylidene]-acetohydrazide (7c): Yield: 79%; *m.p.* 252-253C°; elemental analysis: anal. calcd. for elemental analysis: anal. calcd. for $C_{24}H_{20}N_2O_2$ (368 g/mol): C, 78.24; H, 5.47; N 7.60; found: C, 78.23; H, 5.48; N, 7.61; UV-Vis (CH_3OH) (λ max): 221.8, 278.8; IR (KBr, cm^{-1}): 3344 m, 3186 m, 3045 m, 3021 m, 2961 m, 2878 m, 1638 s, 1608 s, 1580s, 1569 w, 1513 s; ^{1}H-NMR (300 MHz, DMSO-d6, δ, ppm, *J*, Hz): 11.55 (s, NH, eq); 11.16 (s, NH, eq); 10.90 (s, NH, ax); 10.82 (s, NH, ax); 9.89 (s, OH); 8.11 (s, CH=N, eq); 7.98 (s, CH=N, eq); 7.87 (s, CH=N, eq); 7.71 (s,CH=N, ax); 7.61 (s, CH=N, ax); 6.70-7.70 (m,12H, Harom); 7.04 (2H, s, H$^{10'-11'}$); 4.67 (H$^{5'}$, t, 7.6, ax); 3.80 (H$^{5'}$, t, 7.6, eq); 2.97 (2H, H$^{12'}$, d, 7.6, ax); 2.54 (2H, H$^{12'}$, d, 7.6, eq); ^{13}C-NMR (75 MHz, DMSO-d6, δ, ppm): 172.07 (C=O, eq); 171.83 (C=O, ax); 166.90 (Cq, eq); 165.99 (Cq, ax); 159.32 (Cq-O, ax); 159.22 (Cq-O, ax); 159.05 (Cq-O, eq); 146.33 (CH=N, eq); 146.10 (CH=N, ax); 142.69 (CH=N, ax); 139.90 (Cq, ax); 139.56 (Cq, eq); 134.96 (Cq); 134.92 (Cq, ax); 134.00 (Cq, ax); 133.85 (Cq, ax); 131.32 (CH, eq); 130.81 (CH, ax); 129.54 (Cq, eq); 129.72 (Cq, ax); 128.84 (CH, eq); 128.68 (CH, ax); 128.49 (CH); 129.29 (CH); 127.61 (CH); 126.57 (CH); 126.52 (CH); 125.58 (CH); 125.49 (CH); 125.36 (CH); 125.21 (CH); 127.05 (CH); 122.88 (Cq, ax); 122.89 (Cq, ax); 115.74 (CH, eq); 115.62 (CH, eq); 49.44 (CH, 55.55 ax); 49.26 (CH, equatorial ax); 49.42 (CH, ax); 35.29 (CH$_2$, ax); 31.32 (CH$_2$, eq)

Cytoxicity evaluation

The newly compounds were tested for potential cytotoxicity using an alternative methods on invertebrate organisms, *Daphnia magna*. This test is rapid, simple and cost-efficiently and can predict biological activities such as anticancer and analgesic activities [11, 18-23].

Daphnia magna bioassay

Biological determinations were performed in duplicate and 1% DMSO was used as negative control. The experiment was carried out in PP wells with 10 organisms at a volume of 4 mL/sample. From each compound, 6 concentrations ranging from 0.25 - 20 μg/mL were tested. The viability was determined at 24 and 48 h and the lethality curves were plotted against the logarithm of concentrations and lethality (%). The prediction was performed with the

Table I. Results of *Daphnia magna* bioassay

Compound	Predicted $LC50_{24h}$ (µg/mL)	95% CI for predicted $LC50_{24h}$ (µg/mL)	Determined $LC50_{24h}$ (µg/mL)	95% CI for determined $LC50_{24h}$ (µg/mL)	Determined $LC50_{48h}$ (µg/mL)	95% CI for determined $LC50_{48h}$ (µg/mL)
7a	57.3	1.09-3000	28.59	NC	20.56	NC
7b	17.2	0.879-338	NC	NC	21.62	17.22 - 27.15
7c	21	1.54-287	7.58	6.11 - 9.35	5.36	4.83 - 5.95

LC50 - 50% lethal concentration; 95% CI - 95% confidence interval ; NC - not calculated due to the obtained results

Nano-Lazar software application and could only be performed at 24h. The assay results are shown in table 1.

Statistical analysis

The lethal concentrations that kill 50% of organisms (LC50) were determined by interpolating on lethality - logarithm of concentration curves using the least squares fit method. 95% confidence intervals of LC50 (CI95%) and the correlation coefficients (r^2) of the curves, were also calculated. All calculations were performed using GraphPad Prism version 5.0 software (USA).

Results and discussions

Chemistry

The addition of 2-(5*H*-dibenzo[a,d][7]annulen-5-yl)acetohydrazides to aromatic aldehydes is confirmed in the infrared spectra of the new acylhydrazones **7a-c** by the appearance of a new absorption bands due to stretching vibration of C=N imine group (1608-1622 cm^{-1}). In the IR spectra of the acylhydrazones **7a-c**, the bands appearing at 1638-1663 cm^{-1} are attributed to the characteristic amide I $\nu_{C=O}$ band. Also the amide ν_{NH} stretching band of these compounds is observed in the IR spectra at 3181 - 3457 cm^{-1}.

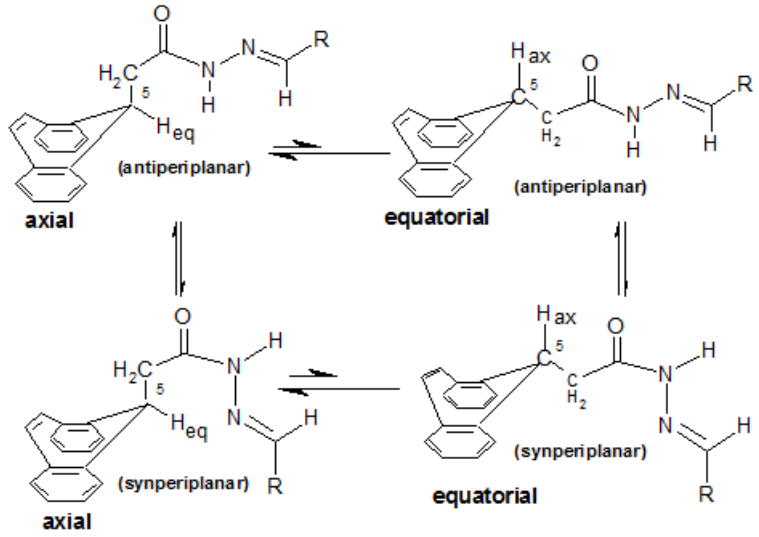
The new N-acylhydrazones **7a-b** present four conformational isomers confirmed by ^{1}H-NMR spectra (scheme 2). Because of the assemblage of imine functions the N-acylhydrazones may exist as C=N double bond stereoisomers (E/Z), but using ^{1}H-NMR and ^{13}C-NMR data, Palla and co-workers posited that N-acylhydrazones compounds are present in solution as the E geometric isomer, which is less sterically hindered compared to the Z form [24]. Because of the dibenzo[a,d][7]annulene moiety the newly compounds may exist as axial/equatorial conformers which are interconvertible by middle ring inversion and because of the the amide CO-NH bond the N-acylhydrazones **7a-c** may exist syn/antiperiplanar conformers (scheme 2) [24,25] So, the multiplication of NMR signal has been attributed to the presence of antiperiplanar/synperiplanar and axial/equatorial conformers.

In the spectrum of acylhydrazones **7a-c** the NH protons signals are duplicated and appear as singlets between 10.90-12.01 ppm, because of to the presence of antiperiplanar/synperiplanar and axial/equatorial conformers. The double bond protons $H^{10'}$ and $H^{11'}$ appear as singlets at 7.04-7.06ppm (scheme 3). Aromatic protons of the dibenzo[a,d][7]annulene moiety give a complex signal between 7.20-7.40ppm as well as those derived from the aromatic ring of the aldehyde moiety.

In the ^{1}H-NMR spectrum at axial isomer of the N-acylhydrazone **7a-c** the $H^{5'}$(eq) is deshielded, manifested as a triplet at 4.62-4.68 ppm, whereas the $CH_2^{12'}$ protons are shielded by the double bond, showing a doublet at 2.58-2.97 ppm (scheme 2). Double bonds shields $H^{5'}$- axial, while aromatic rings deshield $H^{5'}$-equatorial, because of the current ring. The $H^{5'}$(ax) appears at δ =3.75-3.80 ppm (triplet) and $CH_2^{12'}$(ax) protons are deshielded by the double bond, showing a doublet at 2.54-2.98 ppm.

In the ^{13}C-NMR spectrum of **7a-c** the C=O and -CH=N- signals are duplicated and appears at 172 ppm (for C=O) and 140-147 ppm (for -CH=N-).

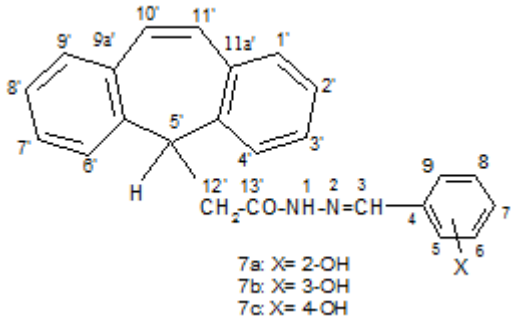
For carbon atoms in the double bond $CH^{10'}$= $CH^{11'}$ the signals are duplicated and appear at= 130.9-131.3 ppm. The rest of the tertiary carbon atoms ($CH_{aromatic}$) give complex signals between =123-139 ppm. Because the carbonyl substituent used in the condensation is donor (OH), signals occur even for more shielded values (115-124 ppm) and also duplications of signals due to axial / equatorial and sin / antiperiplanar isomers.



Scheme 2. General structure and stereochemistry of acylhydrazone **7a-c**

ax = axial isomer

eq = equatorial isomer



Scheme 3. The general structure of 7a-c with atom numbering.

Cytoxicity evaluation

Daphnia magna bioassay

The assay results are shown in table 1. LC50 and 95% confidence intervals for *Daphnia magna* cytotoxicity assay were calculated on 24 and 48 h and compared with the values obtained by *in silico* toxicity prediction. The highest toxicity was exhibited by compound **7c**, followed by **7a** and **7b**, both at 24h and 48h. Compound **7b** was not toxic at all in the first 24h, and compound **7a** produced a toxicity of only 10%. *In silico* prediction toxicity for compounds **7a** and **7b** resulted in lower values at 24h, indicating a high toxicity that was not observed at 24h for the two compounds. Taking into account the 95% confidence intervals obtained by *in silico* analysis, all values determined at 48h fit with within these. Thus, *in silico* 95% confidence intervals are broader and include those calculated on the basis of experimental determinations.

Conclusions

In this paper we described the synthesis, spectral characterization and the cytotoxic activity of new compounds possessing the 5H-dibenzo[a,d][7]annulene moiety from acylhydrazones class. The structures of compounds were confirmed by spectral data (IR-, UV-, ^{1}H-NMR and ^{13}C-NMR).The ^{1}H-NMR spectra of **7a-c** N-acylhydrazones indicated the presence of four conformational isomers (ax/eq, syn/anti).

All the compounds have been investigated for their cytotoxic activity on aquatic invertebrates. Compound **7c** exhibited the highest toxicity to *Daphnia magna* crustaceans and thus high biological activity. The *in silico* prediction toxicity on *Daphnia magna* with Nano Lazar software is useful and offers the advantage of determining test concentrations and the evaluation of 95% confidence intervals of LC50.

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