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Short communication

Synthesis, crystal structure and anticancer activity of novel derivatives of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formate

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Abstract

Synthesis and anticancer activity of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formates (**7–12**) are presented. The title compounds were obtained by two independent synthesis methods from 1-aryl-2-hydrazono-imidazolidines (1-aryl-2-hydrazino-imidazolines) (**1–6**) by cyclocondensation reaction with diethyl 2-(hydroxyimino)malonate (**A**) and diethyl 2-oxomalonate (**B**). Molecular structure of synthesized compounds was confirmed by IR, ^{1}H NMR, EI-MS spectra, elemental analysis and X-ray crystallography for **12**. Compounds **10** and **11** exhibited anticancer activity towards following cancer cells: LS180 (ECACC 87021202, human Caucasian colon adenocarcinoma cells), SiHa (ECACC 85060701, uterus cancer cells), T47D (ECACC 85102201, human breast carcinoma cells). Compound **10** was found to be the most active against SiHa cancer line; its GI was 41 and 52%, respectively in both examined concentrations (10 and 50 μg ml^{-1}), whereas compound **11** had the highest potential to reduce the growth of LS180 and SiHa cancer lines, especially in a higher dose (50 μg ml^{-1}). Moreover, the distinctly marked lower cytotoxicity of tested compounds against normal cell lines (HSF, human skin fibroblast cells and Vero African Green Monkey Kidney cells, GMK clone) and almost two-times higher against cancer cell lines was confirmed. Also antibacterial activity of starting 1-(2-chlorophenyl)-2-hydrazonoimidazolidine hydroiodide (**4**) is presented. Molecular structure of **4** was confirmed by IR, ^{1}H NMR, ^{13}C NMR, EI-MS spectra, elemental analysis and ^{1}H–^{1}H COSY, HMBC and HMQC correlations. The marked antibacterial activity for this compound in relation to *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 with equal minimal inhibitory concentration values of 15.62 and 15.62 μg ml^{-1} was found.

Keywords: Imidazo[2,1-*c*][1,2,4]triazines; Crystal structure; Anticancer activity; 1-aryl-2-hydrazono-imidazolidine; Antibacterial activity

1. Introduction

Compounds containing the 1,2,4-triazine moiety are found in natural sources and many of them showed important biological activities. For instance, azaribine-antiviral drug is structurally based on the 1,2,4-triazine heterocyclic system [1]. Condensed 1,2,4-triazines found application as pharmaceuticals, herbicides, pesticides and dyes [2–6]. Pyrrolo[2,1-*f*][1,2,4]triazines showed an interesting broad spectrum antiproliferative activity and a pronounced in vitro growth inhibitory activity against leukaemic cell lines, comparable to that of 9-deazaadenosine, whilst pyrrolo[2,1-*c*][1,2,4]triazines demonstrated inhibitory effects on the growth of a wide range of cancer cells generally at 10^{-5} M level and in some cases even at micromolar concentrations [7,8]. Some of pyrazolo[5,1-*c*][1,2,4]triazines exhibited antitumor and antifungal activity [9,10]. It is

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noteworthy that many potential anticancer and antiviral drugs have been modeled on them [11–14]. Recently, there is a widespread interest in the synthesis and design of novel imidazo[2,1-*c*][1,2,4]triazine derivatives because of their potential biological activities associated with their skeleton. For instance certain derivatives of imidazo[2,1-*c*][1,2,4]triazin-4(1*H*)-one were synthesized as new bicyclic nucleosides related to 6-azaisocytosine [15].

Previous studies concerning the synthesis of imidazo[2,1-*c*][1,2,4]triazin-4(4*H*)-ones [16–19] carried out in the Department of Synthesis and Technology of Drugs, Medical University of Lublin, have disclosed some compounds with various aryl substituents at position 8, and with benzyl, arylmethyl, methoxycarbonylmethyl and hydroxyl substituents at position 3. These compounds have revealed a significant antinociceptive activity on the central nervous system in behavioral animal tests (the "writhing syndrome" and "hot plate"), and a low acute toxicity. Also, a derivative of imidazo[2,1-*c*][1,2,4]triazin-4(4*H*)-one with a 4-chlorophenyl substituent at the 8 position and a hydroxycarbonylmethyl substituent at the 3 position had significant activity against all Gram-negative bacterial strains tested [20]. Prompted by the biological interest and our previous reports, and continuing our searching for bioactive molecules [16–20] it seemed worthwhile to synthesize some novel biheterocyclic derivatives containing a pharmacophoric 1,2,4-triazine moiety. In this paper we would like to present the preparation and biological activity assessment for ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formate derivatives.

2. Chemistry

Biologically active 1-aryl-2-hydrazono-imidazolidines (1-aryl-2-hydrazino-imidazolines) (**1–6**) as starting materials were prepared by patent pending and according to Sztanke et al. [19]. Treatment of hydrazones **1–6** with diethyl 2-(hydroxyimino)malonate (**A**) or diethyl 2-oxomalonate (**B**) in refluxing *n*-butanol afforded the corresponding ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl) formates (**7–12**) after a ring closure. Reactions of 1-aryl-2-hydrazono-imidazolidine derivatives with diethyl 2-(hydroxyimino)malonate and diethyl 2-oxomalonate have not been reported in the literature as yet and these reactions represent novel methods for preparing the polynitrogenated bicyclic imidazo[2,1-*c*][1,2,4]triazine system. The above mentioned reactions can be carried out starting both from free base of 1-aryl-2-hydrazono-imidazolidine (method *i*) or from its hydroiodide salt in presence of triethylamine (method *ii*) with comparable yields. The reaction conditions were established experimentally. The course of reactions could include the formation of intermediate chain derivatives (2-[(1-aryl-imidazolidin-2-yliden)-hydrazono]malonic acid diethyl esters) as result of condensation of 1-aryl-2-hydrazono-imidazolidines (**1–6**) with diethyl 2-(hydroxyimino)malonate (**A**) and concomitant release of a hydroxylamine molecule or in consequence of condensation reaction of above mentioned 1-arylimidazolidin-2-one hydrazone derivatives with diethyl 2-oxomalonate (**B**) and liberation of a water molecule (Scheme 1).

These intermediates could potentially cyclize to derivatives of the imidazo[2,1-*c*][1,2,4]triazine system of type **7–12** (with concomitant release of an ethanol molecule) or of the imidazo[2,1-*c*][1,2,4]triazole one (with liberation of ethyl formate). Finally, it was found, that the two mentioned methods led to the formation of the imidazo[2,1-*c*][1,2,4]triazine derivatives, as a result of liberation of an ethanol molecule and the ring closure and no trace of the imidazo[2,1-*c*][1,2,4]triazole derivatives were detected. Two general the synthetic routes to imidazotriazine derivatives **7–12** are shown in Scheme 1. Based on the spectral data (IR, NMR, MS) as well as X-ray crystallographic studies, the concurrent course of cyclization reaction to the imidazo[2,1-*c*][1,2,4]triazole derivatives was excluded. In conclusion, the same compounds were obtained by two different synthetic approaches that imply the condensation reaction of appropriate heterocyclic hydrazone derivatives (**1–6**), as well as with diethyl 2-(hydroxyimino)malonate and diethyl 2-oxomalonate. Therefore the structure of these compounds was confirmed by two independent syntheses. Mixed melting points have not shown any depression. The IR, ^{1}H NMR, MS spectral data of these compounds were also identical. Both synthetic pathways in the synthesis of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formate derivatives may be useful in view of biological interest in this class of compounds.

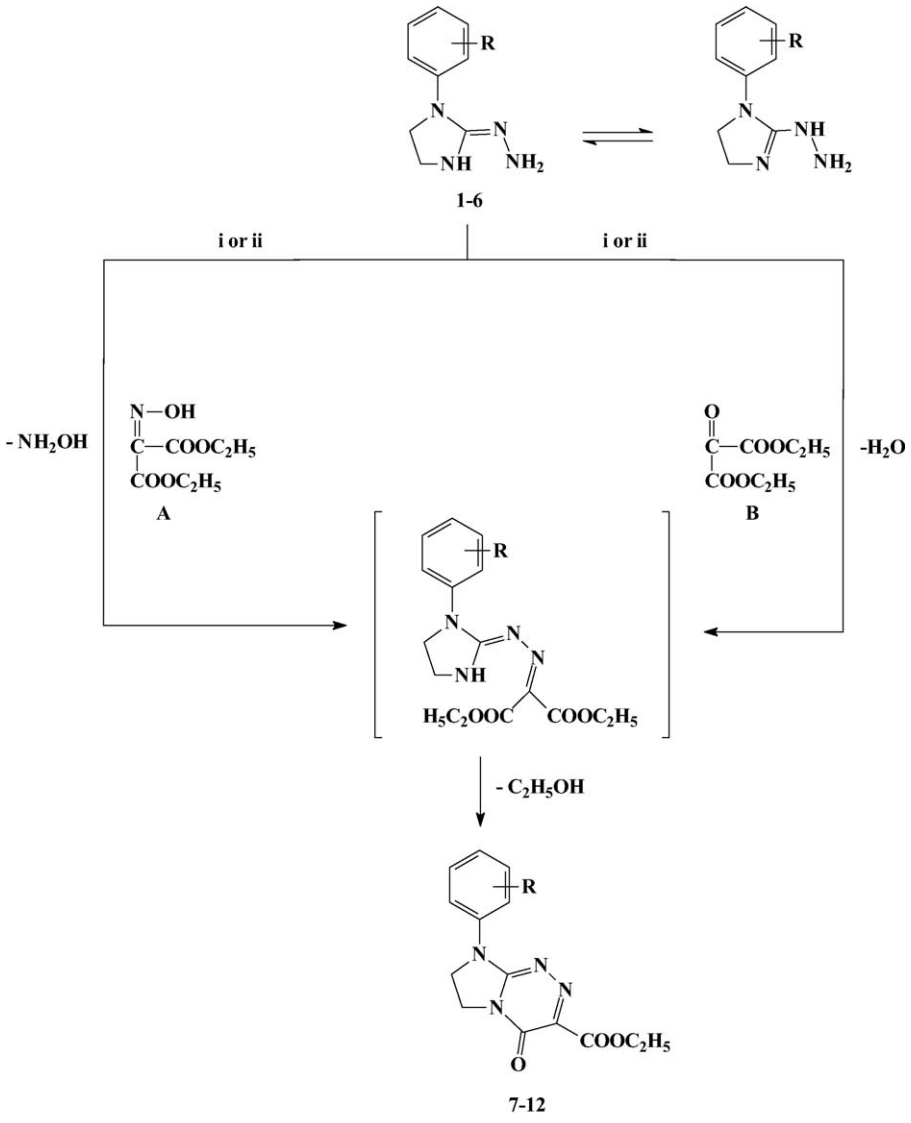
The scrutiny of ^{1}H NMR, IR, MS spectra confirms that under the reaction conditions, formation of the bicyclic imidazo[2,1-*c*][1,2,4]triazine ring system of type **7–12** from acyclic intermediates is accompanied with the liberation of an ethanol molecule. Additionally, the structure of **12** was confirmed by X-ray crystallography. Perspective view of the molecule **12** with atom numbering is shown in Fig. 1.

NMR spectral characteristic of the imidazo[2,1-*c*][1,2,4]triazines (**7–12**) is included in the experimental part.

In the IR spectra of compounds **7–12** the presence of absorption bands in the range of 1684–1689 cm^{-1} and 1552–1561 cm^{-1}, attainable to the triazine-C=O group and the C=N bond at the ring junction, respectively confirmed the formation of cyclic products, whereas the presence of absorption bands at about 1739 cm^{-1} was characteristic for the ester-C=O group derived from the ethoxycarbonyl formation. From the IR spectral data there is a clear relationship between the frequency of the ring carbonyl group -C=O and the frequency of the C=N bond at the ring junction and the difference in their frequencies is of the order of 127–132 cm^{-1}. These data are in complete agreement with the literature data [21].

The chemical and physical data for compounds **7–12** are given in experimental protocols.

Biologically active intermediate 1-(2-chlorophenyl)-2-hydrazonoimidazolidine hydroiodide (**4**) was obtained by a previously reported method [22] but its biological activity, physicochemical and spectral data have not been reported in the literature as yet. Its experimental details are reported in the experimental part. Although in the case of compound investi-



1, 7 : R= 4-CH_3; 2, 8 : R= 4-CH_3O; 3, 9 : R= 2-Cl; 4, 10 : R=3-Cl; 5, 11 : R= 3,4-Cl_2; 6, 12 : R= 2,6-Cl_2

i = (1-6) + (A) or (B), *n*-butanol, reflux, 7h;

ii = (1-6) ·HI + (A) or (B), triethylamine, *n*-butanol, reflux, 7h

Scheme 1. Synthetic route to obtained compounds.

gated the hydrazone-hydrazine tautomerism is possible but in solution (DMSO-d_6) only the presence of the hydrazone tautomer was observed. In the ^1H–^1H COSY spectrum of free base of **4** the correlation between endocyclic-NH proton signal derived from the imidazolidine ring at position 3 and the H-4 methylenic protons signals were observed as expected for the hydrazone tautomer. Also in the HMBC NMR spectrum of this compound there is a lack of correlation between the exocyclic-NH proton (–*NH*NH_2) from possible hydrazinic structure and the C-2 carbon. The HMBC and HMQC NMR spectroscopic data for compound **4** are presented in the Table 1. NMR spectral characteristic of compound **4** is given in the experimental protocols. The chemical shift values of the C-5 and C-4 carbon atoms exhibit unequal character as well (51.3 ppm for C-5 and 41.1 ppm for C-4) as was seen in the ^{13}C NMR spectrum.

3. Pharmacology

3.1. Antimicrobial studies

Determination of the in vitro antimicrobial activity of the compound **4** was performed using the microdilution method, according to the National Committee for Clinical Laboratory Standards (NCCLS) [23,24] and the disc-diffusion method by Kirby-Bauer [25,26].

The in vitro activities of the obtained compound against pathogenic bacteria (reference strains and clinical isolates), yeast-like fungi and moulds were compared. The microdilution method for estimation of MIC values (the lowest concentration of compound required to inhibit the growth of the tested microorganism) was applied to evaluate the antibacterial activity. In

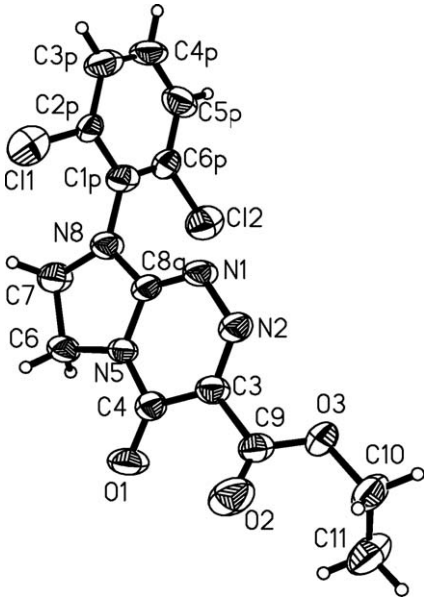
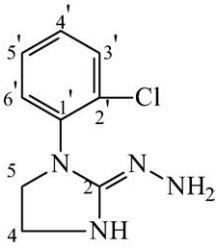


Fig. 1. An ORTEP [36] drawing of the molecule **12**. Bond distances within the heterocyclic ring system are: N1–N2 1.366(5); N2–C3 1.312(6); C3–C4 1.455 (7); C4–N5 1.357(6); N5–C6 1.476(6); C6–C7 1.524(7); C7–N8 1.462(6); C8a–N8 1.345(6); N1–C8a 1.285(6); C8a–N5 1.379(6); C4–O1 1.225(6) Å.

Table 1
The HMBC and HMQC NMR spectroscopic data for free base of compound **4**



	HMBC correlations	HMQC correlations
C-2	H-4, H-5	–
C-4	H-4, H-5	H-4, H-5 (w)
C-5	H-4, H-5	H-5, H-4 (w)
C-1′	H-6′, H-4′ (w), H-5′ (w)	–
C-2′	H-3′	–
C-3′	H-4′, H-5′	–
C-4′	H-3′, H-6′	–
C-5′	H-6′, H-4′ (w)	H-5′
C-6′	H-4′, H-5′	H-6′

w, weak correlation.

this method two reference strains of bacteria—*Staphylococcus aureus* ATCC 25923 (Gram-positive bacteria) and *Escherichia coli* ATCC 25922 (Gram-negative bacteria) were included in this study. The antibacterial potency of the compound **4** under conditions was compared with the activities of topical antibacterial drugs—ampicillin and chloramphenicol.

3.2. Anticancer activity studies

The newly synthesized imidazotriazines of type **10** and **11** were evaluated for their anticancer activity towards three human tumor cell lines derived from various cancer types (colon, uterus, breast): LS 180 (ECACC 87021202, human Caucasian colon adenocarcinoma cells), SiHa (ECACC 85060701, uterus cancer cells), T47D (ECACC 85102201, human breast carcinoma cells). Besides two normal cell lines were included in the cytotoxicity study: HSF (human skin fibroblast cells—primary cell line) and Vero (ECACC 88020401, African Green Monkey Kidney cells, GMK clone).

3.3. Behavioral assays

The effect of the investigated compound **10** in behavioral animal studies was carried out on male Albino Swiss mice.

4. Results and discussion

The antimicrobial activities of the starting 1-(2-chlorophenyl)-2-hydrazonoimidazolidine hydroiodide (**4**) against bacterial, moulds and yeast-like fungi strains and the MIC values against two reference bacterial strains (*S. aureus* ATCC 25923 and *E. coli* ATCC 25922) were tested by using the disc-diffusion and the microdilution assay. The results from experiments were compared with those of ampicillin and chloramphenicol as references for antibacterial agents. The MIC values of the compound tested and both standard drugs are listed in Table 3.

The compound tested (**4**) in the present study was found to have highly significant antibacterial activities against the microorganisms listed in Table 2 but no antifungal activities. The examined compound was also inactive against moulds

Table 2
Antimicrobial activities of evaluated compound **4** against the tested bacterial and fungal isolates using the disc diffusion method

Microorganisms	Compound **4**		Standard
	I	II	
E. coli ATCC 25922	+	+	++
Pseudomonas aeruginosa	+	+	–
Proteus vulgaris	+	+	–
Klebsiella pneumoniae	–	–	–
Enterobacter aerogenes	–	–	–
S. aureus ATCC 25923	+	+	++
Staphylococcus epidermidis	+	+	+
Streptococcus pyogenes	–	–	++
Streptococcus agalactiae	–	–	++
Candida albicans	–	–	–
Aspergillus sp.	–	–	–

I, concentration of 100 μg ml^{-1}; II, concentration of 200 μg ml^{-1}. Zones of growth inhibition in: –, +, ++. – : 0–10 mm (R, resistant); + :11–16 mm (I, intermediate susceptible); ++ : 17–25 mm (S, susceptible). Standard: ampicillin.

Table 3
Antibacterial activity expressed as MIC (μg ml^{-1}) of tested compound **4**

Microorganisms/code	Compound	Standards	
	4	**A**	**C**
E. coli ATCC 25922	15.62	12.5	3.91
S. aureus ATCC 25923	15.62	12.5	3.91

Standards: A, ampicillin; C, chloramphenicol.

and yeast-like fungi. The 1-(2-chlorophenyl)-2-hydrazonoimidazolidine hydroiodide (**4**) showed good activity in relation to reference bacterial strains (*S. aureus* ATCC 25923 and *E. coli* ATCC 25922). This compound was effective against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 at concentrations of 15.62 μg ml^{-1} and 15.62 μg ml^{-1}, respectively. The tested compound was found to exhibit a comparable level of activity to ampicillin. Its antibacterial potency was fourfold lower than that of chloramphenicol (Table 3). Compound **4** was also found to exhibit activity against *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Staphylococcus epidermidis* in concentrations of 100 and 200 μg ml^{-1} in the disc-diffusion assay (Table 2).

As a result, compound **4** was found to exhibit potent in vitro antibacterial activity with MIC value of 15.62 μg ml^{-1} against *S. aureus* ATCC 25923 and may be considered promising for the development of new antibacterial agents.

Compounds **10** and **11** were evaluated for their anticancer activity. Results for each test compounds are reported as the growth inhibition percentage of the tested cells in comparison to untreated ones. Compounds which reduced growth are passed on for evaluation towards three human cancer and two normal cell lines. Compounds **10**, **11** were found to be active. According to the data listed in the Table 4 compounds **10** and **11** have potential to reduce the growth of the uterus cancer cell line (SiHa), colon adenocarcinoma cell line (LS180) and breast carcinoma cell line (T47D). Compound **10** was the most active against SiHa cancer line, because its GI was 41 and 52%, respectively for both examined concentrations (10 and 50 μg ml^{-1}). Compound **11** was found to be the most potent against LS180 and SiHa cancer lines, especially in a higher concentration (50 μg ml^{-1}). Its GI values were 50 and 46% for these cancer lines, respectively. Based on performed examination, the distinctly marked lower cytotoxicity of tested compounds against normal cell lines and almost two-times higher against cancer cell lines was ascertained. Taking into consideration the GI comparative study results concerning the influence of tested compounds on cancer and normal cell lines it can be expected the selective action of examined compounds. Also the anticancer activity of tested compounds seemed to be dose-dependent.

Table 4
Inhibition of in vitro normal and tumor cells growth by imidazotriazines **10**, **11**

Cell line	Cytotoxicity (GI in %)			
	10		**11**	
	I	II	I	II
Normal cell lines				
HSF	16	17	14	20
GMK	20	25	20	25
Cancer cell lines				
LS180	30	35	30	50
SiHa	41	52	30	46
T47D	26	41	13	28

HSF, human skin fibroblast cells—primary cell line; Vero (GMK, ECACC 88020401, African Green Monkey Kidney cells); LS180 (ECACC 87021202), human Caucasian colon adenocarcinoma cells; SiHa (ECACC 85060701), uterus cancer cells; T47D (ECACC 85102201), human breast carcinoma cells; I, concentration of 10 μg ml^{-1}; II, concentration of 50 μg ml^{-1}.

In pharmacological animal tests compound **10** showed no effect on the central nervous system of mice in behavioral tests applied. It has been found that the investigated compound had no antinociceptive properties. This compound given in a dose of 0.1 LD_{50} did not produce a decrease in a number of animals exhibiting pain reactivity in the "writhing syndrome" test. Its acute toxicity was relatively low (over 1000 mg kg^{-1} i.p.). Moreover compound **10** given in a dose of 0.1 LD_{50} did not impair the motor coordination of mice in the rota-rod test.

In conclusion, the tested imidazo[2,1-*c*][1,2,4]triazine derivatives (**10**, **11**) demonstrate antiproliferative properties those warrant further investigation as potential anticancer agents. Further studies are in progress to define the important mechanisms of action of above mentioned compounds.

5. Experimental protocols

5.1. Chemical and X-ray crystal structure analysis

Chemicals (hydrazine hydrate, diethyl 2-(hydroxyimino) malonate, diethyl 2-oxomalonate) were purchased from Merck and Sigma-Aldrich as 'synthesis grade' and used without further purification. Melting points (m. p.) were determined on a Boetius apparatus and are given uncorrected. The IR spectra were measured as potassium bromide pellets using a Perkin-Elmer 1725X spectrometer. ^{1}H NMR spectra for compounds **7**, **8**, **10** were recorded on a Bruker 200 MHz spectrometer in DMSO-d_6. ^{1}H NMR spectra of the other ones (**4**, **9**, **11**, **12**) were recorded on a Bruker 300 MHz spectrometer in DMSO-d_6 with TMS as an external standard at 295 K. ^{13}C NMR spectrum for compound **4** was recorded on a Bruker AC 200F instrument. Besides ^{1}H–^{1}H COSY, HMBC and HMQC correlations were made for this compound. Mass spectroscopic analyses for compounds **7–9** were performed on an AMD-402 and for compounds **11–12** on Trace DSQ mass spectrometers for molecular ion peaks.

Diffraction data for **12** were measured at 295 K on a KM4 diffractometer using variable scan speed in the ω-2θ scan mode and graphite monochromated CuKα radiation (λ = 1.54178 Å). A single crystal of dimensions 0.42 × 0.21 × 0.20 mm was selected from the sample crystallized from DMF/methanol (4:1) mixture. Thin-layer chromatography was carried out on commercial Merck SiO_2 60 F_{254} plates with toluene/ethyl acetate/methanol (1:3:0.5) eluent system and visualized in UV light λ = 254 nm and 355 nm. Elemental analyses were performed on a Perkin–Elmer analyzer and were in range of ±0.5% for each element analyzed (C, H, N, Cl, I). The starting 1-(2-chlorophenyl)-2-hydrazonoimidazolidine hydroiodide (**4**) was obtained by earlier described method [19,22] but its physicochemical and spectral data have not been described in the literature as yet and are the following:

1-(2-Chlorophenyl)-2-hydrazonoimidazolidine hydroiodide (**4**). Recrystallization from propan-2-ol, yield 65%, m.p. 223–225 °C. Analysis for $C_9H_{12}ClIN_4$: IR (KBr) (ν, cm^{-1}): 1511 (N^+H), 1570 (C=N), 3457–3313 (NH + NH_2); ^{1}H NMR (δ, ppm, DMSO-d_6 , TMS): 3.77 (dd, J = 9.3 Hz, J' = 8.1 Hz, 2H,

CH_2), 4.02 (dd, $J = 9.3$ Hz, $J' = 8.1$ Hz, 2H, CH_2), 4.94 (s, 2H, NH_2), 7.47–7.70 (m, 4H, $CH_{arom.}$), 8.76 (s, 1H, NH), 9.39 (s, 1H, N^+H); ^{13}C NMR (δ, ppm, DMSO-d_6 , TMS) 41.1 (imidazolidine-C-4), 51.3 (imidazolidine-C-5), 128.9 (C-5′), 130.5 (C-6′), 130.6 (C-2′), 131.1 (C-3′), 132.2 (C-4′), 133.0 (C-1′), 159.1 (imidazolidine-C-2); *m/z*: 338[M^+].

5.1.1. Synthesis of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)formates (method i) (general procedure)

Free base of 1-aryl-2-hydrazonoimidazolidine [22] (0.05 mol) was dissolved in 80 ml of *n*-butanol. Diethyl 2-(hydroxyimino)malonate (9.45 g, 0.05 mol) was added and the mixture was heated under reflux for 7 h. During that time precipitation of the solid started. The mixture was cooled overnight and the precipitate yielded was collected and purified by recrystallization from DMF or DMF/methanol in the proportion indicated.

5.1.2. Synthesis of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)formates (method ii) (general procedure)

Diethyl 2-(hydroxyimino)malonate (9.45 g, 0.05 mol) was added to the suspension of appropriate 1-aryl-2-hydrazonoimidazolidine hydroiodide [22] (0.05 mol) in 70 ml of *n*-butanol. The mixture was stirred and triethylamine (5 ml) was added. The reaction was carried out under reflux for 7 h. During that time precipitation of solid started. The crude product obtained after cooling was collected, washed off with cold methanol and finally purified by recrystallization from DMF or DMF/methanol in the proportion indicated.

5.1.3. Synthesis of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)formates (method i) (general procedure)

Free base of 1-aryl-2-hydrazonoimidazolidine [22] (0.05 mol) was dissolved in 80 ml of *n*-butanol. Diethyl 2-oxomalonate (8.71 g, 0.05 mol) was added and the mixture was heated under reflux for 7 h. During that time precipitation of the solid started. The mixture was cooled overnight and the precipitation yielded was collected and purified by recrystallization from DMF or DMF/methanol in the proportion indicated.

5.1.4. Synthesis of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)formates (method ii) (general procedure)

Diethyl 2-oxomalonate (8.71 g, 0.05 mol) was added to the suspension of appropriate 1-aryl-2-hydrazonoimidazolidine hydroiodide [22] (0.05 mol) in 70 ml of *n*-butanol. The mixture was stirred and triethylamine (5 ml) was added. The reaction was carried out under reflux for 7 h. During that time precipitation of solid started. The crude product obtained after cooling was collected, washed off with cold methanol and finally purified by recrystallization from DMF or DMF/methanol in the proportion indicated.

*5.1.4.1. Ethyl 1-[4-oxo-8-(4-methylphenyl)-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]formate (**7**).* Recrystallization from DMF; yield 61% (from reaction with A)/63% (from reaction with B) (method *i*), 55% (from reaction with A)/52% (from reaction with B) (method *ii*), m.p. 205–208 °C. Analysis for $C_{15}H_{16}N_4O_3$: IR (KBr) (ν, cm^{-1}): 1736 (ester –C=O), 1687 (triazine –C=O), 1560 (C=N); ^1H NMR (δ, ppm, DMSO-d_6 , TMS): 1.29 (t, $J = 7.1$ Hz, 3H, $-OCH_2CH_3$), 2.32 (s, 3H, CH_3), 4.18 (t, $J = 3.1$ Hz, 4H, $2CH_2$), 4.30 (q, $J = 7.1$ Hz, 2H, $-OCH_2CH_3$), 7.27 (d, $J = 8.6$ Hz, 2H, ar: H-2′ and H-6′), 7.70 (d, $J = 8.6$ Hz, 2H, ar: H-3′ and H-5′); EIMS [70 eV, *m/z* (%)]: 300 (M^+, 75.37), 256 (84.29), 228 (43.70), 200 (100.00), 172 (22.43), 145 (67.56), 91 (42.89).

*5.1.4.2. Ethyl 1-[4-oxo-8-(4-methoxyphenyl)-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]formate (**8**).* Recrystallization from DMF/methanol (2:1) mixture; yield 67% (from reaction with A)/59% (from reaction with B) (method *i*), 60% (from reaction with A)/57% (from reaction with B) (method *ii*), m.p. 149–151 °C. Analysis for $C_{15}H_{16}N_4O_4$: IR (KBr) (ν, cm^{-1}): 1740 (ester –C=O), 1689 (triazine –C=O), 1561 (C=N); ^1H NMR (δ, ppm, DMSO-d_6 , TMS): 1.29 (t, $J = 7.1$ Hz, 3H, $-OCH_2CH_3$), 3.78 (s, 3H, OCH_3), 4.17 (t, $J = 3.1$ Hz, 4H, $2CH_2$), 4.29 (q, $J = 7.1$ Hz, 2H, $-OCH_2CH_3$), 7.04 (d, $J = 9.1$ Hz, 2H, ar: H-2′ and H-6′), 7.72 (d, $J = 9.1$ Hz, 2H, ar: H-3′ and H-5′); EIMS [70 eV, *m/z* (%)]: 316 (M^+, 100.00), 272 (21.85), 216 (92.95), 161 (42.98), 107 (4.71).

*5.1.4.3. Ethyl 1-[4-oxo-8-(2-chlorophenyl)-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]formate (**9**).* Recrystallization from DMF/methanol (3:1) mixture; yield 57% (from reaction with A)/54% (from reaction with B) (method *i*), 53% (from reaction with A)/57% (from reaction with B) (method *ii*), m.p. 190–192 °C. Analysis for $C_{14}H_{13}ClN_4O_3$: IR (KBr) (ν, cm^{-1}): 1742 (ester –C=O), 1686 (triazine –C=O), 1555 (C=N); ^1H NMR (δ, ppm, DMSO-d_6 , TMS): 1.29 (t, $J = 7.1$ Hz, 3H, $-OCH_2CH_3$), 4.14–4.25 (m, 4H, $2CH_2$), 4.31 (q, $J = 7.1$ Hz, 2H, $-OCH_2CH_3$), 7.26–8.12 (m, 4H, ar-H); EIMS [70 eV, *m/z* (%)]: 320 (M^+, 42.93), 276 (100.00), 248 (52.90), 220 (83.39), 165 (59.57), 111 (39.20).

*5.1.4.4. Ethyl 1-[4-oxo-8-(3-chlorophenyl)-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]formate (**10**).* Recrystallization from DMF/methanol (3:1) mixture; yield 65% (from reaction with A)/60% (from reaction with B) (method *i*), 59% (from reaction with A)/63% (from reaction with B) (method *ii*), m.p. 189–191 °C. Analysis for $C_{14}H_{13}ClN_4O_3$: IR (KBr) (ν, cm^{-1}): 1741 (ester –C=O), 1684 (triazine –C=O), 1552 (C=N); ^1H NMR (δ, ppm, DMSO-d_6 , TMS): 1.29 (t, $J = 7.1$ Hz, 3H, $-OCH_2CH_3$), 4.17–4.26 (m, 4H, $2CH_2$), 4.32 (q, $J = 7.1$ Hz, 2H, $-OCH_2CH_3$), 7.25–8.12 (m, 4H, ar-H); EIMS [70 eV, *m/z* (%)]: 320 (M^+, 26.83), 276 (100.00), 248 (44.86), 220 (72.93), 165 (31.40), 111 (31.09).

*5.1.4.5. Ethyl 1-[4-oxo-8-(3,4-dichlorophenyl)-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]formate (**11**).* Recrystalli-

zation from DMF/methanol (4:1) mixture; yield 67% (from reaction with A)/62% (from reaction with B) (method *i*), 66% (from reaction with A)/57% (from reaction with B) (method *ii*), m.p. 248–250 °C. Analysis for $C_{14}H_{12}Cl_2N_4O_3$: IR (KBr) (ν, cm^{-1}): 1738 (ester –C=O), 1687 (triazine –C=O), 1555 (C=N); ^{1}H NMR (δ, ppm, DMSO-d_6 , TMS): 1.29 (t, J = 7.1 Hz, 3H, -OCH_2CH_3), 4.13–4.27 (m, 4H, $2CH_2$), 4.31 (q, J = 7.1 Hz, 2H, –OCH_2CH_3), 7.71–8.27 (m, 3H, ar-H); EIMS [70 eV, *m/z* (%)]: 354 (M^+, 35.76), 310 (71.85), 309 (34.13), 282 (48.26), 254 (100.00), 253 (76.47), 245 (9.02), 228 (11.29), 226 (11.69), 220 (4.05), 219 (3.41), 218 (11.22), 212 (6.33), 201 (26.17), 199 (44.84), 192 (11.77), 186 (11.26), 174 (69.48), 173 (39.55), 172 (50.82), 159 (8.38), 145 (36.85), 133 (10.59), 124 (10.67), 111 (14.50), 109 (30.39), 81 (58.93), 75 (15.28), 70 (21.88).

*5.1.4.6. Ethyl 1-[4-oxo-8-(2,6-dichlorophenyl)-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl]formate (**12**)*. Recrystallization from DMF/methanol (4:1) mixture; yield 59% (from reaction with A)/61% (from reaction with B) (method *i*), 54% (from reaction with A)/58% (from reaction with B) (method *ii*), m.p. 232–234 °C. Analysis for $C_{14}H_{12}Cl_2N_4O_3$: IR (KBr) (ν, cm^{-1}): 1737(ester –C=O), 1686 (triazine –C=O), 1554 (C=N); ^{1}H NMR (δ, ppm, DMSO-d_6 , TMS): 1.29 (t, J = 7.1 Hz, 3H, –OCH_2CH_3), 4.03–4.43 (m, 4H, $2CH_2$), 4.32 (q, J = 7.08 Hz, 2H, –OCH_2CH_3), 7.53–7.72 (m, 3H, ar-H); EIMS [70 eV, *m/z* (%)]: 354 (M^+, 7.20), 319 (33.94), 310 (100.00), 309 (58.84), 282 (50.49), 254 (61.57), 253 (91.14), 245 (24.18), 220 (18.95), 219 (18.98), 218 (7.40), 212 (14.12), 201 (9.30), 199 (15.69), 192 (54.84), 186 (9.18), 174 (64.39), 173(23.32), 172 (46.49), 159 (12.00), 145 (17.80), 133 (15.60), 124 (12.76), 111 (17.36), 109 (30.63), 81 (17.46), 75 (19.58), 70 (38.33).

Crystal data for **12**: $C_{14}H_{12}Cl_2N_4O_3$, FW = 355.18, monoclinic, space group $P2_1/c$, a = 11.502(2) Å, b = 7.296(1) Å, c = 18.574(4) Å, α = 90°, β = 105.86(3)°, γ = 90°, V = 1499.4 (5) $Å^3$, Z = 4, d_{calc} = 1.573 g cm^{-3}, μ(CuKα) = 4.095 mm^{-1}. In the θ range 4.95–80.31°, 3369 reflections were collected and corrected for absorption. The structure was solved by direct methods using SHELXS-93 [27] program. The refinement was performed by full-matrix least-squares on F^2 using 3264 unique reflections (R_{int} = 0.051) and SHELXL-97 [28] program. The non-hydrogen atoms were refined with anisotropic displacement parameters. H-atom positions were located from the geometry and were given isotropic factors of 1.2 U_{eq} of the bonded C-atoms; the C–H bond 'riding' model was used in the refinement. For 209 parameters refined, final discrepancy factors are R_1 = 0.0412, wR_2 = 0.0999 for 746 reflections with $I > 2\sigma(I)$, and R_1 = 0.3192, wR_2 = 0.1612 for all data, S = 0.901.

Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre as CCDC No. 275239. Copies of the data can be obtained on application to The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (Fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).

5.2. Microbiology

5.2.1. Disc diffusion assay

Assay of antimicrobial activity in vitro. Compound (**4**) was tested for its antimicrobial (antibacterial and antifungal) activities by disc-diffusion method by Kirby-Bauer, using Mueller–Hinton medium for bacteria and the same medium with 4% glucose for fungi. The majority of test microorganisms were obtained from clinical specimens of the Laboratory of Medical Microbiology Department, Medical University of Lublin. The assayed collection included the following microorganisms: *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus agalactiae* (Gram-positive bacteria), *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* (Gram-negative bacteria), *Candida albicans*, *Aspergillus spp*. Besides, two reference strains of bacteria—*S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were included in these studies.

In the disc-diffusion method, sterile paper discs (φ 5 mm) impregnated with dissolved in DMSO compound at concentrations of 100 μg ml^{-1} and 200 μg ml^{-1} were used. Discs containing DMSO were used as control. The microorganisms cultures were spread over the following appropriate media: Mueller–Hinton agar for *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and Sabouraud agar for the yeast-like fungi (*Candida albicans*) and for the moulds (*Aspergillus spp*.) in Petri dishes. Then, the paper discs impregnated with the solutions of the compound tested (**4**) were placed on the surface of the media inoculated with the microorganism. The plates were incubated at 35 °C per 24 h for the microorganisms cultures. After incubation, the growth inhibition zones around the discs were observed indicating that the examined compound inhibits the growth of microorganism [25,26]. Each assay in this experiment was repeated three times.

Ampicillin was used as a standard drug. DMSO was used as solvent control. Results were interpreted in terms of the diameter of the inhibition zone and are shown in Table 2.

5.2.2. Microdilution assays

The minimal inhibitory concentration (MIC) values for compound tested (**4**), defined as the lowest concentration of the compound preventing the visible growth, were determined by using microdilution broth method according to NCCLS standards [23]. The inocula of microorganisms (10^6 cfu ml^{-1}) were prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The test compound dissolved in DMSO was first diluted to the highest concentration (500 μg ml^{-1}) to be tested. Then serial twofold dilutions were made in concentration ranges from 1.95 to 500 μg ml^{-1} in 10 ml sterile tubes. A prepared suspension of the standard microorganisms was added to each dilution in a 1:1 ratio. Growth (or its lack) of microorganisms was determined visually after incubation for 24 h at 37 °C. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC.

The minimal inhibitory concentration (MIC) values were studied for reference bacterial strains: *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 towards compound **4** determined in the disc-diffusion assay. Ampicillin and chloramphenicol were used as a standard drugs for comparison in the antibacterial study. Control experiments using DMSO were done for antibacterial activity studies. The presented results were obtained from three independent measurements. The investigations were carried out in the Department of Medical Microbiology, Medical University, Lublin.

5.3. Inhibition of tumor cell growth assay

Compounds **10**, **11** were selected for in vitro screening towards three human cancer cell lines: human Caucasian colon adenocarcinoma (LS180), human uterus cancer (SiHa) and human breast carcinoma (T47D). Besides two normal cell lines: human skin fibroblasts (HSF) and Vero (Green Monkey Kidney cells) were included in the cytotoxicity study.

In the current protocol each cell line was inoculated at 10^4 cells per ml density and preincubated on a microtiter plate. Test agents were then added at double examined concentrations (10 and 50 μg ml^{-1}) and culture incubated for 72 h. End-point determinations were made with 5-bromo-2′-deoxy-uridine (BrdU) labeling [29–32] on Elisa reader (BIO-TEC Instruments USA).

The growth percentage was evaluated spectrophotometrically versus untreated controls with used cell viability of growth assay. Results for each spectrophotometrical measure were noticed as percent of growth inhibition. All experiments were done in triplicates. The investigations were carried out in the Department of Biology and Genetics, Medical University, Lublin.

5.4. Behavioral experiments

Behavioral experiments were performed on male Albino Swiss mice (body weights of 20–25 g) purchased from licensed dealer, Górzkowska, Warsaw, Poland. The animals were kept in 8–10 to a cage, at room temperature of 20 ± 1 °C, on a 12/12 h light/dark cycle (light on from 7 a.m. to 7 p.m.). Standard food (Bacutil, Motycz, Poland) and water were available ad libitum. Experimental and control groups consisting of 10 animals each were selected by means of a randomized schedule. The experiments were performed between 8 a.m. and 3 p.m. The investigated compound **10** was administered intraperitoneally (i.p.) as suspension in 3% Tween 80 in a constant volume of 10 ml kg^{-1}. Controls animals received the equivalent volume of solvent. Each experimental group consisted of ten animals.

The investigations were carried out in the Department of Toxicology, Medical University, Lublin, Poland.

5.4.1. Acute toxicity in mice for compound *10*

Acute toxicity was assessed according to Litchfield and Wilcoxon methods [33] and presented as LD_{50} calculated from the mortality of mice after 24 h.

5.4.2. The influence of compound *10* on the motor impairment in mice

Motor coordination was evaluated in the rota-rod test [34].

5.4.3. Pain reactivity in the "writhing syndrome" test in mice for compound *10*

Pain reactivity was measured in mice by the "writhing syndrome" test of Witkin et al. [35]. The test was performed in mice by the intraperitoneally injection of 0.6% solution of acetic acid in a volume of 10 ml kg^{-1} 30 min after the administration of the investigated compound **10** in a dose of 0.1 LD_{50}. The number of writhing episodes was counted for 30 min after the injection of acetic acid.

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