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Short communication

Antiviral activity of the products of cyclization of dimethyl 2-[(1-arylamino-1-arylmethylidene)hydrazono]succinate

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Abstract – In this research, conditions of cyclization of dimethyl 2-[(1-arylamino-1-arylme-thylidene)hydrazono]succinate **1**–**5** leading to the formation of 3,4-diaryl-5-carboxymethyl-1,2,4-triazole **6**–**10** and methyl 2-(5-oxo-3,4-diaryl-1,4,5,6-tetrahydro-1,2,4-triazine-6-ylidene)acetates **11**–**15** and the biological activity of some of them have been examined. Their chemical structures were confirmed by IR, ^{1}H-NMR, EI-MS and elemental analysis. Substances **8** and **13** exhibited moderate virucidal activity and partially inhibited absorption of the viruses to the susceptible cells. The acute toxicity of compounds **6**–**10** was established. For compounds **8** and **13**, the influence on the central nervous system of mice and rats in behavioral tests was examined. © 2001 Éditions scientifiques et médicales Elsevier SAS

3,4-diaryl-1,2,4-triazolo-5-carboxylates / 2-(3,4-diaryl-5-oxo-1,2,4-triazine-6-ylidene)acetates / cytotoxicity / antiviral activity / vesicular stomatitis virus / encephalomiocarditis virus / adenovirus / in vitro study

1. Introduction

Viral infections have for a long time been considered as non-affectable by chemoterapeutics due to innate association of viruses with normal cell structures and biochemistry. Starting from early 1950 with the discovery of antiviral activity of some thiosemicarbazones [1], further findings were launched very fast. Antiviral chemotherapy began with the advent of acyclovir in 1977 as the first specific antiviral agent [2]. With the very fast development of molecular biology of viruses and their biochemistry, it was possible to fight them on the different stages of their activity (cell absorption and penetration, replication) and every year new biological targets are recognized. It results in findings of much better treatments for some viral diseases, especially HIV. The new results in the search for antiviral agents are still presented in scientific literature. Some new active compounds are structurally based on the 1,2,4-triazole (Ribavirine, Vibunazole) or 1,2,4-triazine (Azaribine) heterocyclic systems [3, 4]. This paper presents the results of investigation of antiviral activity of representatives of both 1,2,4-triazole and 1,2,4-triazine groups: 3-(2-pyridyl)-4-phenyl-5-carboxymethyl-1,2,4-triazole (**8**) and methyl 2-(5-oxo-3-(2-py-ridyl)-4-phenyl-1,4,5,6-tetrahydro-1,2,4-triazine-6-ylidene)-acetates (**13**) were synthesized in our laboratory. Their virucidal action and effects on the replication of the selected laboratory strain viruses RNA (VSV, EMCV) and DNA (AV-5) were investigated. Some behavioral pharmacological tests (acute toxicity and ''writhing test'') were also performed to check the influence of the compounds on the central nervous system of the laboratory animals.

2. Chemistry

In has been found that the course of reaction of cyclic amidrazones with dimethyl acetylene dicaboxylate (DMAD) depends on the reaction conditions. It

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has been reported previously that, when this reaction is carried out in the presence of triethylamine, derivatives of 5-hydroxy-1,2-pyrrole and 5-oxo-1,2,4-triazine are formed [5]. We found that carrying out this reaction in high-boiling solvents (e.g. n-butanol) in the absence of base can lead to different products.

The cyclization reaction of dimethyl 2-[(1-arylamino-1-arylmethylidene)hydrazono]succinate when performed in boiling n-butanol can lead to the formation of derivatives of 1,2,4-triazole-5-carboxylic acid **6**–**10** (with concomitant liberation of a molecule of methyl acetate) and 5-oxo-1,2,4-triazine-6-carboxylic acid **11**–**15** (with the liberation of a methanol molecule) with ratio of ca. 1:1. Separation of both reaction products was possible based on their different solubility in ethyl ether. More detailed data for compounds **11**–**15** are given in ref. 6.

In IR spectra, compounds **6**–**10** exhibit characteristic absorption bands corresponding to the carbonyl of the amid function in the range of 1702–1729 cm^{-1}. In their ^{1}H-NMR spectra, only the presence of a singlet of the CH_3 ester group (in 3.3–3.6 ppm range) and a multiplet of the aromatic hydrogens (in 7.3–8.3 ppm range) were observed. In contrast, in IR spectra of compounds **11**–**15**, absorption bands of the carbonyl of the amide group are present in the 1698–1735 cm^{-1} range. In their ^{1}H-NMR spectra, besides a singlet of the CH_3 ester group (in the same range as for compounds **6**–**10**) and a multiplet of aromatic hydrogens (in 7.1–8.2 ppm range), the presence of single signal of N1 hydrogen in the range of 11.2–11.5 ppm was observed (*table I*). Further confirmation of the structures of compounds **8** and **13** was based on their ^{13}C, ^{15}N spectra and X-ray analyses [7].

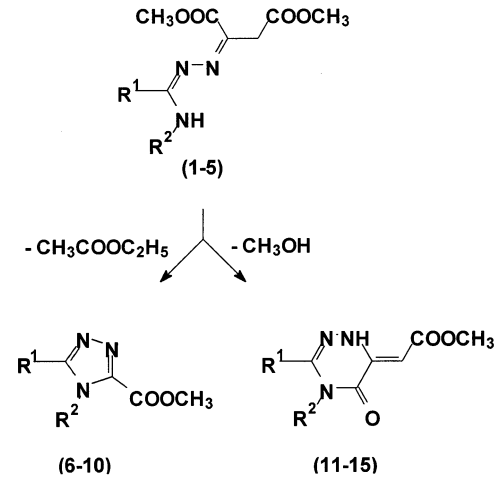
To investigate other ways of cyclization, which can possibly lead to the formation of 1,2,4-triazole derivatives with higher yield, three different media were used in the cyclization reaction. According to the method proposed by Le Count and Greer [5], the respective succinate derivatives were refluxed in (a) water, (b) acetic anhydride and (c) alloy bath without any solvent (ca. 90 °C). It was found that all these methods lead directly to the formation of only the compounds **11**–**15**, but with lower yields than when the reaction is performed in methanol in the presence of triethylamine [6]. The course of the reaction is presented in *figure 1*.

3. Biological investigations

Human adenovirus type 5 (AV-5) received from the National Institute of Hygiene (Warszawa) was propagated in HeLa cells and titrated in human skin fibroblasts (HSF). Vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) strain col MM were obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw. VSV and EMCV were propagated and titrated in L929 cells. The influence of these compounds on acute toxicity (LD_{50}) and in behavioral studies was carried out on male and female albino Swiss mice and male Wistar rats.

4. Results and discussion

The toxicity of the **8** and **13** derivatives were dependent on dose and time of incubation (*figures 2–5*). Moreover, the toxicity of both substances depended



	R^1	R^2
1, **6**, **11**	C_6H_5	C_6H_5
2, **7**, **12**	C_6H_5	p-$CH_3C_6H_4$
3, **8**, **13**	2-C_5H_4N	C_6H_5
4, **9**, **14**	2-C_5H_4N	p-$CH_3C_6H_4$
5, **10**, **15**	2-C_5H_4N	p-$NO_2C_6H_4$

Figure 1. Condition-dependent reactions of dimethyl 2-[(1-arylamino-1-arylmethylidene)-hydrazono]succinate.

Table I. Physicochemical and spectral properties of compounds **6–10**.

Comp. no.	R^1	R^2	Formula m.w.	M.p. (°C)	Yield (%)	IR absorption in KBr (cm^{-1})					1H-NMR (ppm, TMS)		
						CH arom	CH aliph.	CO	C = N	NO_2	CH_3 (s)	OCH_3 (s)	H arom. (m)
6	C_6H_5	C_6H_5	$C_{16}H_{13}N_3O_2$ 279,30	210–212	38	3080	—	1702	1590	—	—	3.7	7.3–7.5
7	C_6H_5	p-CH_3-C_6H_4	$C_{17}H_{15}N_3O_2$ 293,33	182–184	32	3085	2900	1708	1580	—	2.4	3.5	7.1–8.2
8[a]	2-C_5H_4N	C_6H_5	$C_{15}H_{12}N_4O_2$ 280,29	178–180	35	3052	—	1729	1583	—	—	3.7	7.4–8.3
9	2-C_5H_4N	p-CH_3-C_6H_4	$C_{16}H_{14}N_4O_2$ 294,32	168–170	30	3058	2919	1735	1580	—	2.5	3.7	7.0–8.2
10	2-C_5H_4N	p-NO_2-C_6H_4	$C_{15}H_{11}N_5O_4$ 325,29	220–222	37	3090	—	1740	1606	1306	—	3.5	7.0–8.2

[a] EI-MS; 280 (30.1 M^+), 279 (100), 247 (12.5), 167 (15.7), 77(12.9).

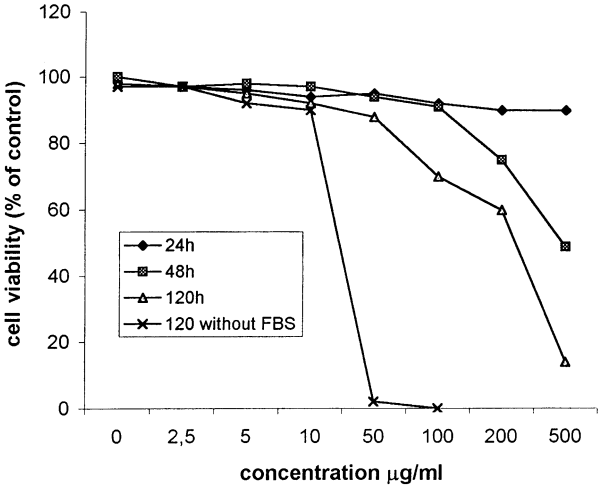


Figure 2. Toxicity of compound **8** for HSF cells.

significantly on the presence of serum. When medium supplemented with 1% fetal calf serum (FCS) was used, the toxicity of derivatives was several times lower than that in medium without FCS (*figures 2* and *3*). The compounds studied are likely to show similar interactions with serum proteins such as Vratizoline®, used as a medicine against herpes viruses [8]. Both derivatives used were more toxic for L929 cells than for HSF; however, both of them at concentrations of 50 μg ml^{-1} were non-toxic for HSF and partially toxic for L929 cell cultures.

The experiments revealed that both substances possessed virucidal activity and during 1 h of contact

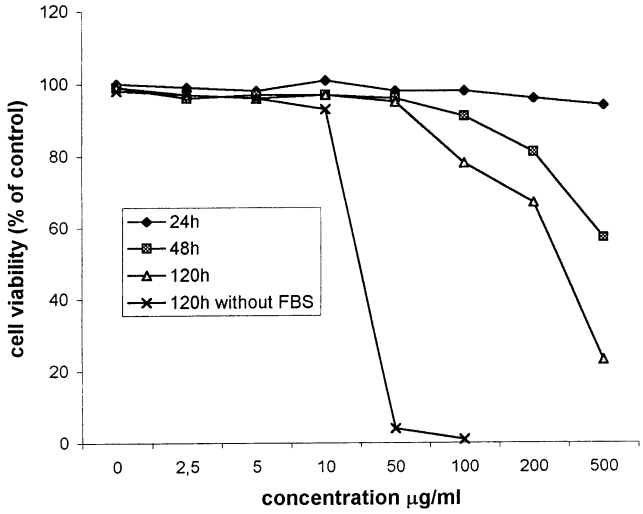


Figure 3. Toxicity of compound **13** for HSF cells.

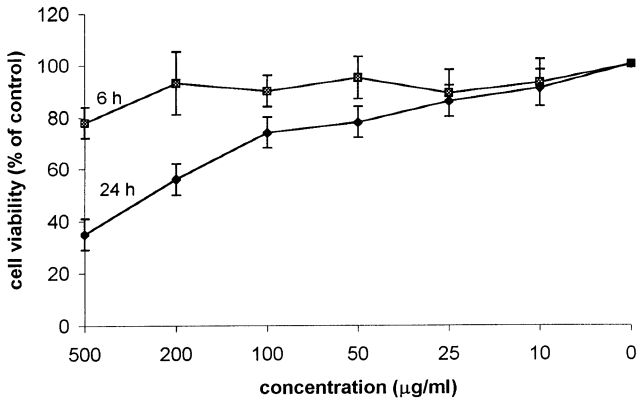


Figure 4. Toxicity of compound **8** for L929.

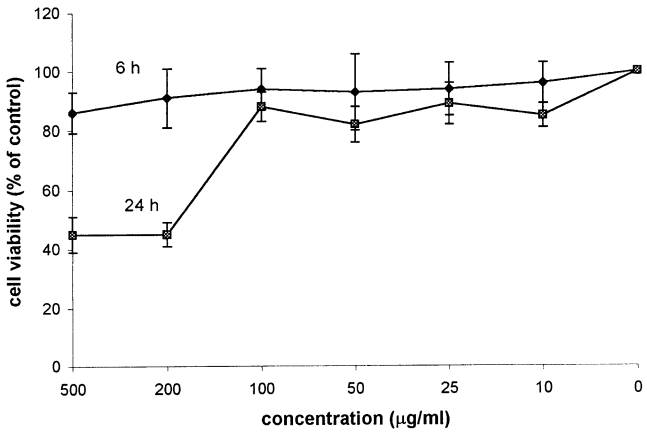


Figure 5. Toxicity of compound **13** for L929.

with virus caused the decrease in titers of viruses by 0.5–1.67 log (*table II*). Moreover, they were active against viruses that belong to different families: Adenoviridae (AV-5), Rhabdoviridae (VSV) and Picornaviridae (EMCV).

Both derivatives examined present in culture medium during all steps of virus life-cycle (from adsorption to release) inhibited AV-5 replication and decreased final virus yield by 0.83 log (**13**) and 1 log (**8**) (*table III*). It seems likely that both substances can inhibit early steps of viral replication, as their presence during adsorption and penetration of viral life-cycle decreased the final yield of EMCV by 0.59 log (**13**) and the final yield of VSV by 1.77 log (*table IV*). The level of inhibition of virus titer for compound **8** was about 10% and, for compound **13,** about 37%. When the influence of both derivatives on the eclipse phase of viral replication (after adsorption and penetration) was examined, no influence of both derivatives on the final yield of VSV and EMCV was observed (*table V*).

4.1. Pharmacological tests

Acute toxicity by accessing LD_{50} dose according to the Wilcoxone and Litchfield method was investigated in the Department of Pharmacology and Toxicology, Medical University, Lublin, Poland. Acute toxicity of compounds **6**–**10** in mice was low and was over 1750 mg kg^{-1} i.p. when that of compounds **11**–**15** (published in the paper [6]) was over 2000 mg kg^{-1} i.p. In behavioral tests, compound **8** exhibited only weak analgesic activity in the "writhing test" when compound **13** showed no effect on the central nervous system of mice in all behavioral tests applied.

Compound **13** from this group also exhibited moderate antibacterial and antifungal activity with MIC values of 200–300 μg ml^{-1} against *Escherichia coli*, *Brucella abortus*, *Mycobacterium smegmatis*, *Candida albicans* and *Epidermophyton floccosum* [6]. Compounds **8** and **13** at a 500 μg ml^{-1} concentration did not affect the microflora of the human digestive tract [9] nor, at a

Table II. Virucidal activity of **8** and **13** derivatives.

	Virus titer ($CCID_{50}$ ml^{-1})*					
Virus	VSV		EMCV		AV-5	
Derivative (μg ml^{-1})	**8**	**13**	**8**	**13**	**8**	**13**
0 (control)	8.00 ± 0.45	8.00 ± 0.45	8.33 ± 0.36	8.33 ± 0.36	4.00 ± 0.45	3.67 ± 0.32
25	7.00 ± 0.45	7.33 ± 0.32	7.67 ± 0.37	7.67 ± 0.37	3.77 ± 0.48	3.00 ± 0.32
50	7.00 ± 0.45	7.33 ± 0.36	7.67 ± 0.37	7.67 ± 0.37	3.00 ± 0.45	2.50 ± 0.32
100	7.00 ± 0.45	7.50 ± 0.41	7.50 ± 0.32	7.67 ± 0.37	2.57 ± 0.32	2.00 ± 0.45

* Incubation of virus with derivatives – 1 h at 37 °C. The virus titers are shown in log ± SD.

Table III. The influence of derivatives on AV-5 replication in HSF.

Derivative	Concentration (μg ml^{-1})	Virus titer ($CCID_{50}$ ml^{-1})
8	0	5.50 ± 0.32
	5	5.00 ± 0.45
	25	4.67 ± 0.37
	50	4.50 ± 0.32
13	0	5.50 ± 0.41
	5	5.00 ± 0.45
	25	4.77 ± 0.48
	50	4.67 ± 0.37

10–400 μg ml^{-1} concentration, the morphotic elements of the green monkey kidney cells [10].

5. Experimental protocols

5.1. Chemistry

Melting points measured on Boetius apparatus are given uncorrected. ^{1}H-NMR spectra were recorded on a Tesla BS 567A (100 MHz) apparatus in D_6-DMSO with TMS as an external standard. IR spectra were recorded on a Specord IR-75 spectrometer. Results of elemental analysis for C, H, and N by the method of microanalysis (performed in the Department of Organic Chemistry, Medical University in Lublin) were in accordance with calculated values (±0.7% for C, 0.75% for N, and 0.9% for H).

The purity of the obtained compounds was examined by the TLC method. TLC was performed on 10×20-cm pre-coated plates Si 60 F_{254} (E. Merck). The silica plates were activated for 30 min at 110 °C. Compounds were dissolved in ethanol or acetone. Samples (1 μl at 1 mg ml^{-1}) were spotted on the plates. The evolution was carried out in Chromdes (Lublin, Poland) horizontal sandwich DS-chamber [11, 12] with binary eluent consisting of a mixture of chloroform and ethyl acetate (70:30 or 75:25). The plates were dried and spots were visualized under UV light at $\lambda = 254$nm.

*5.1.1. General procedure for preparation of compounds **6–10***

Compounds **1–5** (0.01 mol) were dissolved in 30 cm^3 of n-butanol and refluxed for 20 h. After this time, solvent was removed under reduced pressure; the residue was washed several times with ethyl ether and finally purified by crystallization from ethanol.

*5.1.2. General procedure for preparation of compounds **11–15***

A quantity of 0.01 mol of compound **1–5** was refluxed in 30 cm^3 of water for 4 h. Solution was concentrated to a volume of 10 cm^3. The precipitate was formed. After collection and purification by crystallization, the compounds **11–15** were obtained in a yield of 50–60%; 0.01 mol of compound **1–5** was refluxed in 30 cm^3 of acetic anhydride for 6 h. Solvent was removed under reduced pressure and residue was purified by crystallization (yield 70%); 0.01 mol of compound **1–5** was heated in the alloy bath in a temperature of ca. 100 °C for 10 h. Formed precipitate was collected and purified by crystallization (yield 50%); ethyl ether from combined extracts mentioned in 5.1.1 was distilled off and the residue was purified by crystallization from ethanol (yield 30–40%).

5.2. Virucidal investigations

Toxicity for cell cultures and antiviral activity of compounds **8** and **13**.

Table IV. The influence of derivatives on viral adsorption and penetration.

Derivative	Concentration (μg ml^{-1})	Virus titer ($CCID_{50}$ ml^{-1})	
		VSV	EMCV
8	0	4.77 ± 0.48	3.67 ± 0.37
	50	4.33 ± 0.36	3.67 ± 0.37
13	0	4.77 ± 0.48	3.67 ± 0.37
	50	3.00 ± 0.45	3.00 ± 0.45

Table V. The influence of derivatives on eclipse phase of viral replication.

Derivative	Concentration (μg ml^{-1})	Virus titer ($CCID_{50}$ ml^{-1})	
		VSV	EMCV
8	0	8.00 ± 0.45	8.23 ± 0.49
	50	8.33 ± 0.36	8.50 ± 0.32
13	0	8.00 ± 0.45	8.23 ± 0.49
	50	8.00 ± 0.45	8.50 ± 0.32

5.2.1. Cell cultures

A strain of HSF was obtained by standard trypsynization of a 23-year-old woman skin fragment (1 mm^2). HSF were routinely grown in DMEM (Dulbecco's modified Eagle's medium, Gibco) supplemented with 10% FCS (Gibco), 100 μg ml^{-1} of streptomycin, 100 U ml^{-1} of penicillin in plastic tissue culture flasks (Nunc, Denmark).

HeLa cells (human cervix carcinoma) and mouse cell line L929 were obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw. They were grown in Eagle's Minimal Essential Medium (MEM, Sigma), supplemented with 10% FCS and 100 μg ml^{-1} of streptomycin and 100 U ml^{-1} of penicillin. Cultures were incubated at 37 °C in humidified atmosphere with 5% CO_2.

5.2.2. Toxicity in cell cultures

Both substances examined were dissolved in dimethylsulfoxide (DMSO, Sigma) 10 mg ml^{-1}, and then diluted in cell culture media supplemented or not with 1% FCS. HSF or L929 cells were plated into 96-well plastic plates (Nunc, Denmark) at a cell density of 1×10^4 (HSF) or 1×10^5 (L929) cells per well in appropriate media (with 10% FCS) to obtain the confluent growth of cells. After overnight incubation at 37 °C, the media were removed and cells were treated with **8** or **13** derivatives, diluted in media at final concentrations of 500, 200, 100, 50, 25, 10, 5 and 2.5 μg ml^{-1}. Cell cultures were incubated at 37 °C for 24–120 h. The toxicity was estimated by the MTT method according to the assay described by Takenouchi and Munekata [13]. All experiments were done in triplicates.

5.2.3. Virucidal activity

A suspension of viruses was mixed (1:1 v/v) with derivatives **8** or **13**, which were diluted in media without FCS (final concentrations of derivatives: 25, 50 and 100 μg ml^{-1}). The viruses' suspension (VSV, EMCV or AV-5) with media but without derivatives was a control. Mixtures were incubated at 37 °C for 1 h and viruses were titrated in appropriate cell cultures.

5.2.4. The influence on AV-5 replication in HSF

AV-5 virus suspension was serially diluted in MEM supplemented with 1% of FCS and derivatives at final concentrations of 5, 25, 50 μg ml^{-1}. A suspension of AV-5 diluted in medium alone was a virus control. The confluent HSF cells grown in 96-well plastic plates were infected with the diluted virus; immediately after mixing with derivatives, the cytopathic effect of virus (CPE) occurring after 72 h of incubation at 37 °C was estimated under a microscope and the titer of the virus was calculated. The titer of the virus replicating in the presence of the derivatives was compared to the control without derivatives.

5.2.5. The influence on viral adsorption and penetration

Suspension of viruses (VSV and EMCV) was mixed (1:1 v/v) with derivatives at a concentration of 100 μg ml^{-1} (final concentration 50 μg ml^{-1}) prepared in MEM without serum. L929 cells were immediately infected with such mixtures and incubated at 37 °C for 1 h. Controls were infected with the virus suspensions mixed with medium alone. Cells after viral adsorption were washed three times with medium to remove the rest of non-absorbed virus and frozen. After thawing, the viruses were titrated in L929 cell cultures and the titers of viruses present in cultures treated with derivatives were compared to controls.

5.2.6. The influence on eclipse phase of viral replication

L929 cell cultures were infected with viruses (100 $CCID_{50}$ ml^{-1}), incubated for 1 h at 37 °C (absorption of virus) and washed three times with medium. Infected cell cultures were treated with derivatives (50 μg ml^{-1}) diluted in medium supplemented with 2% of FCS, incubated at 37 °C for 24 h and frozen. Control cells were not treated with derivatives. After thawing, viruses were titrated in L929 cell cultures.

5.3. Pharmacology

5.3.1. Behavioral experiments

Behavioral experiments were carried out on male and female albino Swiss mice (body weights of 20–25 g) and male Wistar rats (200–250 g). Investigated compounds were administered i.p. as suspensions in 3% Tween 80 in a constant volume of 10 ml kg^{-1} in mice and 5 ml kg^{-1} in rats. The compounds were administered in doses equivalent to 1/10 or 1/20 of LD_{50}. Control animals received the equivalent volume of solvent. Each experimental group consisted of eight animals.

*5.3.2. Acute toxicity in mice for compounds **6–10***

Acute toxicity was assessed by the methods of Litchfield and Wilcoxon [14] and presented as LD_{50} calculated from the mortality of mice after 24 h.

*5.3.3. Pain reactivity in the 'writhing syndrome' test in mice for compounds **8** and **13***

Pain reactivity was measured by the 'writhing syndrome' test of Witkin et al. [15]. The test was performed in mice by the i.p. injection of a 3.0% solution of acetic acid in a volume of 10 ml kg^{-1} 60 min after administration of the investigated compounds 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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