Antiviral Activity Evaluation of Pyrazolo[4,3-*e*][1,2,4]triazines

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Summary: The main purpose of this study is to screen synthesized pyrazolo[4,3-*e*][1,2,4]triazine derivatives for their antiviral activity against a panel of DNA and RNA viruses. Minimum cytotoxic and minimum virus-inhibitory concentrations of these compounds were determined.

Introduction

Despite of the wide range of spectroscopic and biological activity the pyrazolo[4,3-*e*][1,2,4]triazines are a less known class in the group of condensed pyrazolotriazines [1-4]. Naturally occurring derivatives of this system were found as extracellular metabolites of cyanobacterium of the class *Pseudomonas fluorescens* var. *pseudoiodinine* and *Nostoc spongiaeforme* [1, 2, 4]. The most important members in this family of naturally purine analogs are pseudoiodinine [4], nostocine A[1] and fluviols A-E[2] (Fig. 1). Smirnova *et al.* studied the biological activity of fluviol A and its methyl derivatives fluviols C and E. The most active appeared to be fluviol E which inhibited the growth of Gram-positive and Gram-negative bacteria [5], and shown activity against fungi [2].

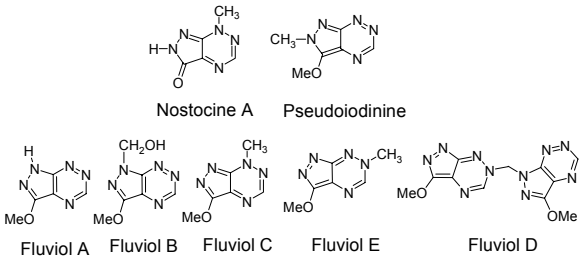


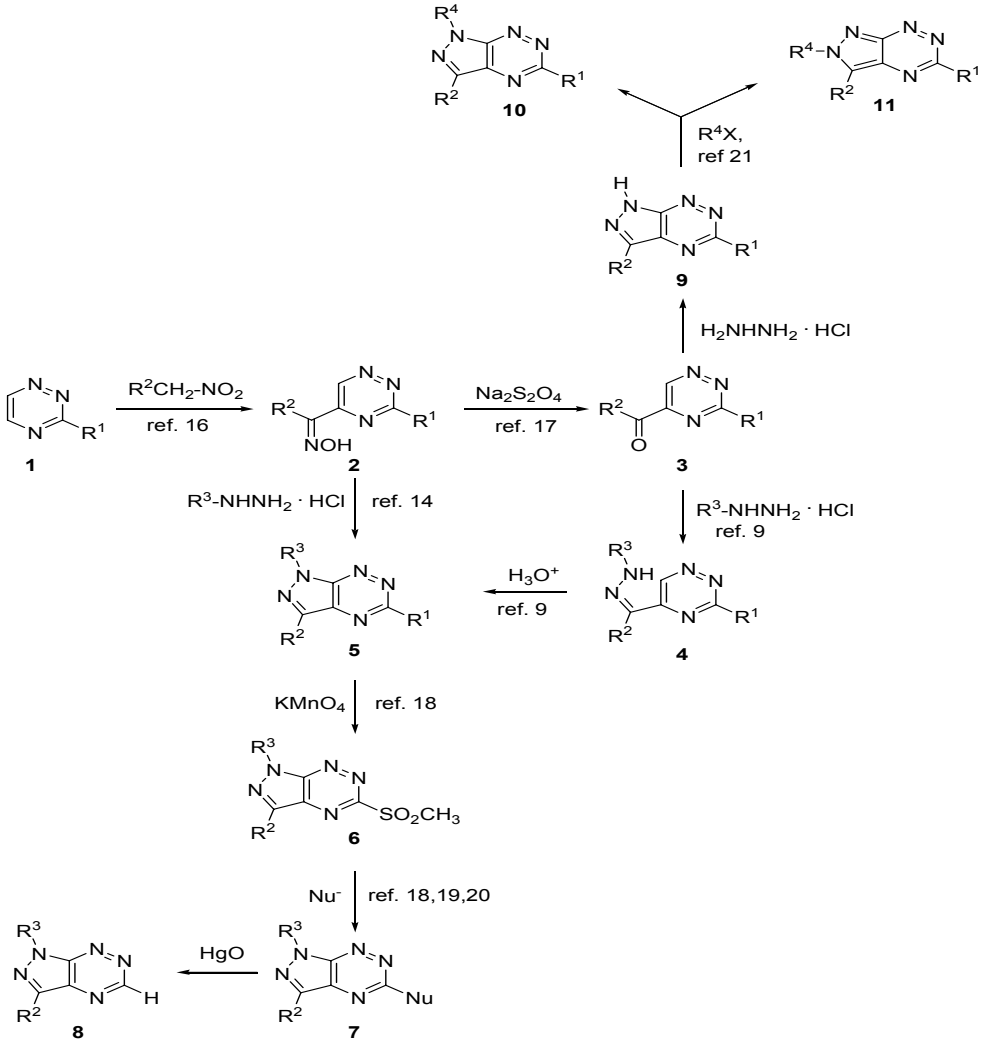
Fig. 1.

The structure of only two of such pigments *e.g*. nostocine A and fluviol A (normethylpseudo-iodinine) have been conclusively determined by x-ray analysis [1, 4]. Moreover, Kelly *et al.* confirmed the structure of nostocine A and fluviol A by total synthesis and this study led to revision of the structure of pseudoiodinine which has been established as fluviol C [6]. There are few different methods described in the literature for the construction of pyrazolo[4,3-*e*][1,2,4]triazine ring. These methods can be divided into two groups, one incorporating the construction of the pyrazole ring onto the 1,2,4-triazine nucleus [7-10] and the second one including the building of the 1,2,4-triazine core on a pyrazole derivative [11-13]. Recently we have published a new approach to the synthesis and functionalization of pyrazolo[4,3-*e*][1,2,4]triazine skeleton [10, 14-16]. Taking into account possible biological usefulness, here we would like to report antiviral activity [17] of pyrazolo[4,3-*e*][1,2,4]triazine derivatives previously synthesized in our laboratory.

Results and Discussion

The synthesis and functionalization of pyrazolo[4,3-*e*][1,2,4]triazine derivatives were achieved by a convenient procedures depicted in Scheme 1. The synthetic route of pyrazolo[4,3-*e*][1,2,4]triazine derivatives started with the synthesis of oximes **2**, which were obtained in the reaction of 3-substituted 1,2,4-triazines **1** with nitroalkanes, according to published procedure [18]. In the next step, the readily available oximes **2** were converted into appropriate ketones **3** in good yields [19], which were subjected to the reaction with hydrazine or its derivatives in the presence of acidic media according to standard procedure to give suitable hydrazones **4** as intermediates for the preparation of pyrazolo[4,3-*e*][1,2,4]triazine derivatives **5** [10, 16]. The resulting hydrazones were converted into a series of ring closured structures **5** under heating with catalytic amount of acid. We found that the mode of cyclocondensation is significantly dependent on the electronic nature of a phenyl ring substituent of the aromatic hydrazones: electron-donating substituents (Me, OMe) favor the cyclization in shorter time contrary to electron-withdrowing substituents (Cl, NO_2) which work favorable for the formation of pyrazolo[4,3-*e*][1,2,4]triazines in longer time. Further,

we investigated high yielding procedure for the preparation of variously substituted pyrazolo[4,3-*e*][1,2,4]triazines **5**, based on the thermally-induced transformation of phenylhydrazones **4** (Scheme 1, R^3 = phenyl or substituted phenyl) in the presence of *p*-toluenesulfonic acid under solvent free conditions [16]. We bring into being that this method offers short reaction time, high yields of products and simple experimental procedure for both pyrazolotriazines with electron-donating and electron-withdrawing groups on the phenyl ring. Oximes of aldehyde **2** (R^1 = Ph, R^2 = H) can react with hydrazine or its derivatives in the presence of concentrated hydrochloric acid to give suitable pyrazolotriazines [15]. Moreover, this method seems to be general and allows introducing the substituents into pyrazole and triazine rings in the first step. We next explored the possibility of structural modification in 5-position of this condensed heteroaromatic ring system using *ipso*-nucleophilic substitution of methylsulfanyl group (R^1=SCH_3) [18]. Important to note, contrary to expectation methylsulfanyl group appeared to be uncreative towards nucleophiles. We have found that more effective nucleofugal group was methylsulfonyl substituent. Thus, sulfide **5** (R^1 = SCH_3, R^2 = CH_3, R^3 = Ph, CH_3, CH_2Ph) was smoothly transformed into corresponding sulfone upon the reaction with potassium manganate (VII) under phase transfer catalysis.



R^1 = Ph, SCH_3
R^2 = H, CH_3, C_2H_5, C_3H_7
R^3 = H, CH_3, Ph, substituted phenyl
Nu = OH, OCH_3, OC_2H_5, $OCH_2CH{=}CH_2$, $OCH_2CH_2CH{=}CH_2$, *o*-cyanophenol, NH_2, $NH\text{-}C_4H_9$, $NH\text{-}CH_2\text{-}Ph$, NH-Ph, S-Ph, CH(COOEt), $NH\text{-}NH_2$
R^4 = CH_3, C_2H_5, C_3H_7, C_4H_9, CH_2Ph

Scheme 1.

Nucleophilic substitution of methylsulfonyl group at the position 5 took place with *O*-, *N*- and *C*-nucleophiles to yield related substitution products in high yields [20, 21]. Oxidation of 5-hydrazino derivative **7** (Nu = NH-NH_2, R^2 = CH_3, R^3 = Ph, CH_3) with yellow mercury (II) oxide in refluxing ethanol gave the 5-unsubstituted pyrazolotriazine **8**. Furthermore, hydrazine function was useful for the preparation of Schiff bases in the reaction with acetone or benzaldehyde [22]. Continuing our study voted to functionalization of pyrazolo[4,3-*e*][1,2,4]triazine core we decided to made use of alkylation method [23]. As depicted in Scheme 1, N_1-unsubstituted derivative **10** was prepared from the previously described 5-acyl-1,2,4-triazine and hydrazine hydrochloride in boiling ethanol. When the reaction took place at room temperature the intermediate compound was isolated, which was *N,N'*-bis-[1-(3-methylsulfanyl-[1,2,4]triazine-5-yl)-ethylidene]hydrazine [21, 22]. Alkylation of the N_1-unsubstituted **10** (R^1 = SCH_3, R^2 = CH_3, R^3 = H) provided a mixture of **11** and **12** in high total yield. The ratio of the isomeric product **11** and **12** depends on the reaction conditions: base and solvent. The structures of evaluated compounds were established by spectroscopic methods and already published elsewhere[9, 14-16, 21-23, 25, 26].

Antiviral Activity

Synthesized previously pyrazolo[4,3-*e*][1,2,4]triazines were evaluated for activity against several RNA- and DNA-viruses, using the following cell-based-assays: (a) Vero cells infected with parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie B4 virus, or Punta Toro virus; (b) human embryonic lung (HEL) fibroblasts infected with herpes simplex virus-1 (KOS), herpes simplex virus-2 (G), acyclovir-resistant herpes simplex virus-1 (KOS ACV^r TK^-), vaccinia virus or vesicular stomatitis virus; (c) human epithelial (HeLa) cells infected with vesicular stomatitis virus, coxsackie B4 virus or respiratory syncytial virus and (d) Madin Darby canine kidney (MDCK) cells infected with influenza virus, subtype A/H1N1, A/H3N2 or B. Results are present in Table-1. As a results of broad spectrum antiviral screening of the derivatives, which had minimal antivirally effective concentration less than one-fifth of minimal cytotoxic concentration, were considered active. No antiviral effects were detected for any tested compound against any of the viruses evaluated.

Table-1: The minimum cytotoxic concentration of pyrazolo[4,3-*e*][1,2,4]triazines.

Compound	R^1	R^2	R^3	R^4	Nu	Minimum cytotoxic concentration (μg/mL)[a]		
						HEL	Vero	HeLa
5a	**Ph**	**H**	**H**			**100**	**100**	**100**
5b	**H**	**CH_3**	**Ph**			**100**	**100**	**100**
5c	**Ph**	**CH_3**	***p*-CH_3-Ph**			**20**	**100**	**100**
5d	**Ph**	**CH_3**	***m*-CH_3-Ph**			**100**	**100**	**100**
5e	**Ph**	**CH_2Br**	**Ph**			**4**	**20**	**20**
5f	**Ph**	**$CHBr_2$**	**Ph**			**4**	**≥4**	**4**
5g	**SCH_3**	**C_2H_5**	***p*-CH_3-Ph**			**100**	**100**	**100**
5h	**SCH_3**	**CH_3**	**CH_3**			**100**	**100**	**≥100**
5i	**SCH_3**	**CH_3**	**tetrahydro-2*H*-pyran-2-yl**			**≥100**	**100**	**>100**
6a		**CH_3**	**tetrahydro-2*H*-pyran-2-yl**			**100**	**100**	**100**
6b		**CH_3**	**CH_2COOMe**			**>100**	**>100**	**>100**
7a		**CH_3**	**Ph**		**$CH(COOEt)_2$**	**20**	**100**	**100**
7b		**CH_3**	**Ph**		**S-Ph**	**100**	**20**	**100**
7c		**CH_3**	**Ph**		***o*-cyanophenol**	**100**	**100**	**100**
7d		**CH_3**	**Ph**		**OH**	**100**	**100**	**100**
7e		**CH_3**	**Ph**		**OCH_3**	**100**	**51**	**100**
7f		**CH_3**	**Ph**		**OC_2H_5**	**20**	**≥20**	**100**
7g		**CH_3**	**Ph**		**$OCH_2CH_2NH_2$**	**20**	**≥20**	**100**
7h		**CH_3**	**Ph**		**NH_2**	**100**	**≥20**	**≥20**
7i		**CH_3**	**Ph**		**NH-Ph**	**20**	**100**	**100**
7j		**CH_3**	**Ph**		**NH-CH_2Ph**	**20**	**100**	**100**
7k		**CH_3**	**Ph**		**NH-$(CH_2)_3CH_3$**	**100**	**100**	**>100**
7l		**CH_3**	**Ph**		**NH-NH_2**	**4**	**100**	**4**
7m		**CH_3**	**Ph**		**NH-N=CHPh**	**20**	**100**	**100**
7n		**CH_3**	**Ph**		**NH-N=$C(CH_3)_2$**	**≥0.8**	**20**	**≥4**
7o		**CH_3**	**CH_2Ph**		**NH-NH_2**	**≥0.8**	**100**	**≥4**
7p		**CH_3**		**CH_3**	**NH-NH_2**	**20**	**20**	**20**
7r		**CH_3**		**CH_2COOMe**	**NH-N=CHPh**	**20**	**20**	**≥20**
8		**CH_3**	**CH_3**			**20**	**20**	**100**
9	**SCH_3**	**CH_3**				**≥100**	**≥100**	**100**
10a	**SCH_3**	**CH_3**		**CH_2COOMe**		**>100**	**>100**	**>100**
10b	**SCH_3**	**CH_3**		***n*-C_3H_7**		**>100**	**100**	**>100**
10c	**SCH_3**	**CH_3**		***n*-C_4H_9**		**100**	**≥20**	**100**
11a	**H**	**CH_3**		**CH_3**		**≥20**	**>100**	**>100**
11b	**SO_2CH_3**	**CH_3**		**CH_3**		**≥20**	**100**	**100**
11c	**OC_2H_5**	**CH_3**		**CH_3**		**100**	**100**	**>100**

Table-1: Continued…

Compound	R^1	R^2	R^3	R^4	Nu	Minimum cytotoxic concentration (µg/mL)[a]		
						HEL	Vero	HeLa
11d	SCH_3	CH_3		C_2H_5		>100	100	>100
11e	SCH_3	CH_3		C_4H_9		>100	100	≥100
11f	SCH_3	CH_3		$CH_2CH_2CH=CH_2$		≥100	100	>100
11g	SCH_3	CH_3		CH_2Ph		100	100	>100
DS-5000						-	>100	>100
(S)-DHPA						-	>250	>250
Ribavirin						>250	>250	>250
Brivudin						>250	-	-
Cidofovir						>250	-	-
Ganciclovir						>100	-	-

[a]Required to cause a microscopically detectable alteration of normal cell morphology.

Antiviral Activity Assay

The compounds were evaluated against the following viruses: herpes simplex virus type 1 (HSV-1) strain KOS, thymidine kinase deficient (TK^-) HSV-1 KOS strain resistant to ACV (ACV^r), Herpes simplex virus type 2 (HSV-2) strains Lyons and G, vaccinia virus Lederle strain, respiratory syncytial virus (RSV) strain Long, Vesicular stomatitis virus (VSV), Coxackie B4, Parainfluenza-3, Reovirus-1, Sindbis, Punta Toro, influenza virus Type A (H1N1, H3N2) and type B and feline corona virus. The antiviral assays were based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) fibroblasts, African green monkey cell (Vero), human epithelial cervix carcinoma cells (HeLa), Crandel feline kidney cells (CFKC) and Madin-Derby canine kidney cells (MDCK). Briefly, confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus, one $CCID_{50}$ being the virus dose to infect 50% of the cell cultures. After a 1-2 h adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds.

Conclusion

The results of antiviral activity of the tested compounds are shown in Table-2. None of the compounds exhibited specific antiviral activity, which means that they did not inhibit replication (formation of viral cytopathogenicity) of any viruses tested at a concentration that was ≥5-fold lower than the minimum cytotoxic concentration.

Acknowledge

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Table-2: Antiviral activity of compounds in HEL, HeLa an Vero cell cultures.

Compound	Min. inhibitory conc. [a] (µg/mL)												
	HEL					Vero					HeLa		
	Herpes Simplex Virus-1(KOS)	Herpes Simplex virus-2 (G)	Vaccinia virus	Vesicular Stomatitis virus	Herpes Simplex virus-1 KOS ACV^r (TK^-)	Para-Influenza-3 virus	Reovirus-1	Sindbis virus	Coxsackie Virus B4	Punta Toro virus	Vesicular Stomatitis virus	Coxsackie Virus B4	Respiratory Syncytial virus
5a	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
5b	>20	>20	>20	>20	>20	>20	>20	>20	>20	20	>20	>20	>20
5c	>4	>4	>4	>4	>4	>20	>20	>20	>20	>20	>20	>20	>20
5d	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
5e	>0.8	>0.8	>0.8	>0.8	>0.8	>4	>4	>4	>4	>4	>4	>4	>4
5f	>0.8	>0.8	>0.8	>0.8	>0.8	>4	>4	>4	>4	>4	>0.8	>0.8	>0.8
5g	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
5h	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>100	>100	>100
5i	>100	>100	>100	>100	>100	>20	>20	>20	>20	>20	>100	>100	>100
6a	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
6b	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
7a	>4	>4	>4	>4	>4	>20	>20	>20	>20	>20	>20	>20	>20
7b	>20	>20	>20	>20	>20	>4	>4	>4	>4	>4	>20	>20	>20
7c	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
7d	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
7e	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	20	>20	>20
7f	>4	>4	>4	>4	>4	>20	>20	>20	>20	>20	>20	>20	>20
7g	>4	>4	>4	>4	>4	>20	>20	>20	>20	>20	>20	>20	>20
7h	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
7i	>4	>4	>4	>4	>4	>20	>20	>20	>20	>20	>20	>20	>20
7j	>4	>4	>4	>4	>4	>20	>20	>20	>20	>20	>20	>20	>20

Table-2: Continued…

Compound	Min. inhibitory conc. [a] (µg/mL)												
	HEL					Vero					HeLa		
	Herpes Simplex Virus-1(KOS)	Herpes Simplex virus-2 (G)	Vaccinia virus	Vesicular Stomatitis virus	Herpes Simplex virus-1 KOS ACV^r (TK$^-$)	Para-Influenza -3 virus	Reovirus-1	Sindbis virus	Coxsackie Virus B4	Punta Toro virus	Vesicular Stomatitis virus	Coxsackie Virus B4	Respiratory Syncytial virus
7k	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>100	>100	>100
7l	0.8	>0.8	>0.8	>0.8	>0.8	>20	>20	>20	>20	>20	>0.8	>0.8	>0.8
7m	>4	>4	>4	>4	>4	>20	>20	>20	>20	>20	>20	>20	>20
7n	>0.8	>0.8	>0.8	>0.8	>0.8	>4	>4	>4	>4	>4	>4	>4	>4
7o	>0.8	>0.8	>0.8	>0.8	>0.8	>20	>20	>20	>20	>20	>4	>4	>4
7p	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
7r	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>20	>20	>20
8	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>20	>20	>20
9	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>20	>20	>20
10a	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
10b	>100	>100	>100	>100	>100	>20	>20	>20	>20	>20	50	>100	>100
10c	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
11a	>20	>20	>20	>20	>20	>100	>100	>100	>100	>100	>100	>100	>100
11b	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
11c	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>100	>100	>100
11d	>100	>100	>100	>100	>100	>20	>20	>20	>20	>20	>100	>100	>100
11e	>100	>100	>100	>100	>100	>20	>20	>20	>20	>20	>100	>100	>100
11f	>100	>100	>100	>100	>100	>20	>20	>20	>20	>20	>100	>100	>100
11g	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>100	>100	>100
DS-5000	-	-	-	-	-	>100	>100	59	>100	9	12	100	0.5
(S)-DHPA	-	-	-	-	-	>250	>250	>250	>250	>250	250	>250	>250
Ribavirin	>250	>250	>250	126	>250	112	>250	>250	>250	112	22	146	29
Brivudin	0.04	126	4	>250	250	-	-	-	-	-	-	-	-
Cidofovir	1	4	3	>250	4	-	-	-	-	-	-	-	-
Ganciclovir	0.06	0.03	>100	>100	2	-	-	-	-	-	-	-	-

[a] Required to reduce virus- induced cytopathogenicity by 50%

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