Full Paper

Synthesis, DNA Binding and Antiviral Activity of New Uracil, Xanthine, and Pteridine Derivatives

Osama I. El-Sabbagh[1], Mohamed E. El-Sadek[1], Samar El-Kalyoubi[1], and Ibrahim Ismail[2]

[1] Department of Medicinal Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt
[2] Veterinary Serum Vaccine Research Institute, Abassia, Cairo, Egypt

Some new 6-amino-1,3-dimethyl-5-(substituted methylidene)aminouracils were synthesized. Most of them were cyclized with triethyl orthoformate as a one-carbon source to afford 1,3-dimethyl-6-substituted pteridine derivatives. Certain uracils gave xanthine instead of the expected pteridine derivatives upon using another one-carbon source such as triethyl orthoacetate or triethyl orthobenzoate. The nucleic acid binding assay revealed that some new compounds showed high affinity, chelation, and fragmentation of nucleic acids whether DNA or RNA contrary to acyclovir that has affinity to DNA only. The antiviral activity of these novel compounds showed that compounds **2e** and **2f** reduced the cytopathogencity of *Peste des petits* ruminant virus (PPRV) on Vero cell culture by 60 and 50%, respectively.

Keywords: Antiviral activity / 6-Amino-1,3-dimethyl-5-(substituted methylidene)aminouracil / Pteridines / Synthesis / Xanthines

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Introduction

Several antiviral agents belong chemically to the uracil derivatives whether nucleosides *e.g.* zidovudine, AZT [1], stavudine, zerit [2] and abacavir, ziagen [3], or non-nucleosides like 1-((2-hydroxyethoxy)methyl)-6-(phenylthio)thymine (HEPT) and its derivatives [4–6]. Other agents which are still used clinically *e.g.* acyclovir are xanthine derivatives. Most uracil derivatives exhibit their antiviral activities by inhibiting the reverse transcription process of the viral replicative cycle and/or by being incorporated into the viral DNA chain, resulting in viral DNA chain termination [7–9].

These facts encouraged us to synthesize new non-nucleoside uracil, xanthine, and pteridine derivatives to evaluate their nucleic-acids binding and antiviral activities.

Correspondence: Dr. Osama I. El-Sabbagh, Department of Medicinal Chemistry, Faculty of Pharmacy, Zagazig University, 44511 Zagazig, Egypt
E-mail: elsabbagh_59@yahoo.com
Fax: +20 5523 03266

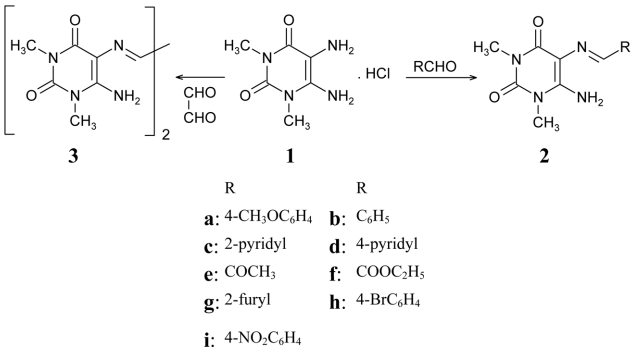
Results and discussion

Chemistry

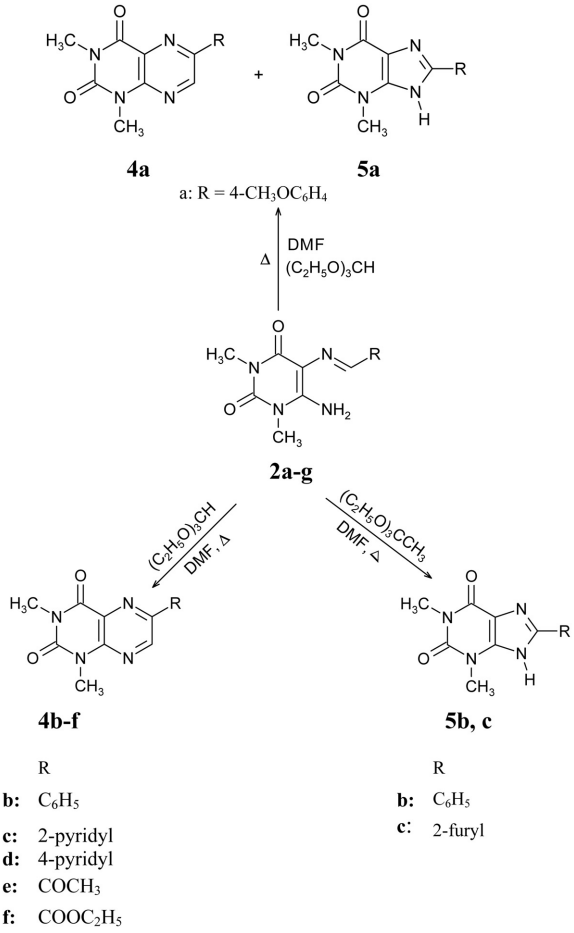
To achieve our target, Schemes 1 and 2 were adopted. The novel 6-amino-1,3-dimethyl-5-(substitutedmethylidene)-aminouracils **2a–i** were obtained with high yields (85–98%) by stirring 5,6-diamino-1,3-dimethyluracil HCl (1) [10–13] with different aromatic or aliphatic aldehydes *e.g.* methyl glyoxal and ethyl glyoxalate hemiacetal in aqueous solution at room temperature for 15 min. This new method is characterized by being more facile and having higher yields as compared to another one reported in the literature [13].

Moreover, *N,N′*-bis-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimid-5-yl)ethylidine diamine **3** was obtained in 64% yield via stirring compound **1** and glyoxal for 20 min using former conditions.

In this work, the novel 1,3-dimethyl-2,4-dioxo-6-substituted-1,2,3,4-tetrahydropteridines **4a–f** were obtained by the intramolecular cyclization of 6-amino-1,3-dimethyl-5-(substituted methylidene)aminouracils **2a–f** using triethyl orthoformate as a one-carbon source and heating the reactants at reflux in DMF.



Scheme 1. Synthesis of uracils **2a–i** and bis-derivative **3**.



Scheme 2. Synthesis of pteridines **4a–f** and xanthines **5a–c**.

The previous data reported [13] that cyclization of the methoxybenzylidene derivative **2a** gave the pteridine derivative **4a** under the former condition as a sole product. But, in the present work, the same reaction yielded a mixture of 6-substituted pteridine **4a** and 8-substituted xanthine **5a** which could be separated by using column chromatography.

On the other hand, upon using another one-carbon sources *e.g.* triethyl orthoacetate or triethyl orthobenzoate to cyclize 5-benzylideneamino- **2b** or 5-(2-furylidene)amino-derivatives **2g** under the previous conditions, the corresponding xanthines derivatives **5b, c** were formed instead of the expected pteridine derivatives. This is attributable to a higher reactivity of triethyl orthoformate as a one-carbon source in comparison with triethyl orthoacetate or triethyl orthobenzoate.

The structure of pteridine and xanthine derivatives were established by ^{1}H-NMR which indicated the disappearance of singlet signal of benzylidenic proton at δ [ppm] = 8.87–9.79 and the singlet integrating two protons of amino groups at δ [ppm] = 7.22–7.63 of the starting Schiff's bases. The spectra showed also the appearance of a singlet at δ [ppm] = 9.18–9.58 corresponded to the proton at C_7-H in case of pteridines and the characteristic singlet at δ [ppm] = 13.56–13.92 for the N_9-H proton of xanthines.

Biological investigation

The newly synthesized compounds were subjected to nucleic acids binding assay using agarose gel electrophoresis method, and, in addition, their *in vitro* antiviral activities were studied.

Nucleic acids binding assay

After electrophoresis, a plasmid DNA band of about 9 kb (1 kb = 1000 bp) and total RNA of about 3 kb were detected in the untreated nucleic acid solution (lane 1, Fig. 1). The addition of dimethylsulphoxide (DMSO) to the nucleic acid mixture was used as negative control, which showed slight fragmentation of DNA and RNA (lane 2). Acyclovir in lane 3 was used as the positive control and showed fragmentation of DNA rather than RNA; the RNA band was clearly identified. Affinity, binding and fragmentation of nucleic acids were discriminated by appearance of nucleic acid smear instead of distinct bands in the gel after electrophoresis [14–16]. Compounds **2a–g** and **4e, f** showed smears for nucleic acids in lanes 4–12 in the agarose gel.

Acyclovir; a synthetic antiviral agent, was chosen for comparison due to its high affinity to DNA binding, chelation, and fragmentation in agarose gel. Compounds **2a–g** and **4e, f** showed a high affinity, chelating, and fragmentation of both DNA and RNA helix in contrary to acyclovir that has affinity to DNA only rather than RNA. These compounds can be considered as a broad spectrum agent of expected antiviral activity. Thus, these compounds were utilized to investigate their antiviral activities while other inactive compounds were excluded.

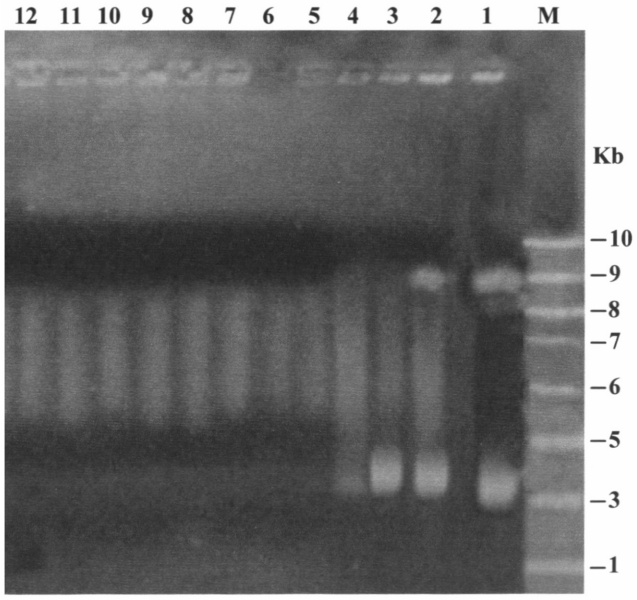


Figure 1. Gel electrophoresis 0.8% w/v agarose of untreated and treated plasmid DNA and RNA. *Lane M*: Molecular weight marker (right side); *Lane 1*: Untreated nucleic acids; *Lane 2:* DMSO treated nucleic acids (negative control); *Lane 3*: Acyclovir treated nucleic acids (positive control); *Lanes 4–12*: Compounds (**2a–g** and **4e, f**) treated nucleic acids.

Antiviral screening

In addition to the nucleic acids binding assay, the antiviral activities of compounds **2a–g** and **4e, f** against *Peste des petits* ruminant virus (PPRV) were examined. PPR virus is a RNA virus that belongs to the Morbilli viruses of the family *paramyxoviridae* [17, 18]. It causes a highly contagious and fatal disease of small ruminants which represents a serious problem in the Middle East.

Firstly, the cytotoxic assays were performed to determine the highest nontoxic concentrations of the tested compounds that could be used [19, 20]. Moreover, for each compound the concentration that reduced the absorbance reading to 50% of the control-well level (CD_{50}) was calculated (Table 1). Subsequently, the antiviral assays were carried out on Vero cell culture which was infected with PPR virus strain; then the tested compounds were added with the infection and one hour after infection in the highest nontoxic concentrations. End point titers (log10 $TCID_{50}$) were determined by evaluating the cytopathic effect (C.P.E) and were calculated by the accumulative method of Reed and Muench [21] and then presented as the% reduction in the cytopathic effects (Table 1).

Table 1 revealed that all the tested compounds exhibited some cytotoxicity and compounds **2e, f** showed the highest % reduction in the cytopathic effects at dose 0.1 mg/mL among all compounds. Cyclization of compounds **2e, f** gave pteridine derivatives **2e, f** which showed weak *in vitro* antiviral activity. The rest of compounds did not show any % reduction in the cytopathic effects at doses up to 0.3 mg/mL.

Table 1. Antiviral and cytotoxic activity of tested compounds **2a–g; 4e, f**.

Compound	Concentration (mg/mL)	% Reduction of C.P.E Time of addition relative to virus (hours)		Cytotoxicity[a] CD_{50} (mg/mL)
		Zero	+1	
Ribavirin[b]	0.001	100	100	0.3
2a	0.3	none	none	0.5
2b	0.3	none	none	>0.6
2c	0.3	none	none	0.4
2d	0.3	none	none	0.4
2e	0.1	60	50	0.5
2f	0.1	50	33	0.5
2g	0.3	none	none	>0.6
4e	0.2	14	14	0.4
4f	0.2	10	8	0.4

[a] CD_{50} values, the concentration of compounds that reduced the absorbance reading to 50% from the control level.
[b] Positive control.

Conclusion

We can conclude that some novel 6-amino-1,3-dimethyl-5-(substituted methylidene)aminouracils were prepared and then cyclized with triethyl orthoformate or triethyl orthoacetate in DMF under reflux conditions giving new pteridines or xanthines. Some tested compounds showed high affinity to nucleic acids whether DNA or RNA upon using agarose gel electrophoresis method and their *in vitro* antiviral activity revealed that compounds **2e** and **2f** reduced the cytopathogencity of *Peste des petits* ruminant virus (PPRV) on Vero cell culture by 60 and 50%, respectively.

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Experimental

Chemistry

All melting points were determined in open glass capillaries on an Electrothermal Mel.-Temp II apparatus (Electrothermal) and are uncorrected. ^{1}H-NMR was recorded on a JEOL JUM-LA 400 MHz spectrometer (JEOL, Tokyo, Japan) using DMSO-d_6 as a solvent and TMS as an internal standard (Chemical shift in δ, ppm). Elemental analyses were performed at the Microanalytical laboratory, Faculty of Chemistry, Konstanz University, Germany and are within ±0.4% of the theoretical values unless otherwise stated. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel-coated aluminium sheets (type 60 F_{254}, Merck, Germany), spots were visualized by iodine vapors or by irradiation with UV light (254 nm). Compound **1** was prepared following the reported procedure [10 – 13].

*General procedure for preparation of compounds **2a – i***

To a solution of 5,6-diamino-1,3-dimethyluracil hydrochloride (**1**, 1.45 mmol) in hot water (20 mL), ammonium hydroxide was added to pH 8. After cooling, the aromatic or aliphatic aldehyde *e.g.* methyl glyoxal or ethyl glyoxalate hemiacetal (1.45 mmol) was added with stirring at room temperature for 15 min The formed product was filtered and crystallized from the proper solvent.

*6-Amino-1,3-dimethyl-5-(4-methoxybenzylidene)-aminouracil **2a***

Yield: 0.38 g (90%), m. p. 197 – 199°C as reported [13] (ethanol). ^{1}H-NMR: δ [ppm] = 3.17 (s, 3H, N_1 – CH_3), 3.40 (s, 3H, N_3-CH_3), 3.80 (s, 3H, OCH_3), 6.95 – 6.98 (d, 2H, ArH), 7.2 (s, 2H, C_6-NH_2), 7.83 – 7.87 (d, 2H, ArH), 9.67 (s, 1H, N=CH). Analysis ($C_{14}H_{16}N_4O_3$) C, H, N.

*6-Amino-5-(benzylidene)amino-1,3-dimethyluracil **2b***

Yield: 0.36 g (94%), m. p. 221 – 223°C as reported [13] (ethanol). ^{1}H-NMR: δ [ppm] = 3.10 (s, 3H, N_1-CH_3), 3.42 (s, 3H, N_3-CH_3), 7.29 – 7.34 (m, 3H, ArH), 7.38 (s, 2H, C_6-NH_2), 7.86 – 7.91 (d, 2H, ArH), 9.78 (s, 1H, N=CH). Analysis ($C_{13}H_{14}N_4O_2$) C, H, N.

*6-Amino-1,3-dimethyl-5-(2-pyrilidene)aminouracil **2c***

Yield: 0.35 g (92%), m. p. 252 – 254°C (DMF/H_2O). ^{1}H-NMR: δ [ppm] = 3.15 (s, 3H, N_1-CH_3), 3.38 (s, 3H, N_3-CH_3), 7.29 – 7.31 (m, 1H, ArH), 7.43 (s, 2H, C_6-NH_2), 7.78 – 7.83 (m, 1H, ArH), 8.38 – 8.41 (d, 1H, ArH), 8.56 – 8.61 (d, 1H, ArH), 9.72 (s, 1H, N=CH). Analysis ($C_{12}H_{13}N_5O_2$) C, H, N.

*6-Amino-1,3-dimethyl-5-(4-pyrilidene)aminouracil **2d***

Yield: 0.37 g (98%), m. p. 263 – 265°C (ethanol). ^{1}H-NMR: δ [ppm] = 3.15 (s, 3H, N_1-CH_3), 3.39 (s, 3H, N_3-CH_3), 7.29 – 7.31 (m, 1H, ArH), 7.55 (s, 2H, C_6-NH_2), 7.83 – 7.85 (d, 2H, ArH), 8.54 – 8.57 (d, 2H, ArH), 9.66 (s, 1H, N=CH). Analysis ($C_{12}H_{13}N_5O_2$), C, H, N.

*5-(Acetylmethylidene)amino-6-amino-1,3-dimethyluracil **2e***

Yield: 0.28 g (85%), m. p. 229 – 230°C (DMF). ^{1}H-NMR: δ [ppm] = 2.36 (s, 3H, CH_3) 3.12 (s, 3H, N_1-CH_3),3.37 (s, 3H, N_3-CH_3), 7.63 (s, 2H, C_6-NH_2), 8.87 (s, 1H, N=CH). Analysis ($C_9H_{12}N_4O_3$) C, H, N.

*6-Amino-1,3-dimethyl-5-(ethoxycarbonylmethylidene)-aminouracil **2f***

Yield: 0.35 g (94%), m. p. 190 – 192°C (ethanol/H_2O). ^{1}H-NMR: δ [ppm] = 1.22 – 1.28 (t, 3H, CH_3), 3.12 (s, 3H, N_1-CH_3), 3.35 (s, 3H, N_3-CH_3),4.14 – 4.23 (q, 2H, CH_2), 7.49 (s, 2H, C_6-NH_2), 8.88 (s, 1H, N=CH). Analysis ($C_{10}H_{14}N_4O_4$) C, H, N.

*6-Amino-1,3-dimethyl-5-(2-furylidene)aminouracil **2g***

Yield: 0.35 g (97%), m. p. 203 – 205°C (ethanol). ^{1}H-NMR: δ[ppm] = 3.16 (s, 3H, N_1-CH_3), 3.36 (s, 3H, N_3-CH_3), 6.57 – 6.59 (m, 1H, ArH), 7.00 – 7.02 (d, 1H, ArH), 7.22 (s, 2H, C_6-NH_2), 7.73 – 7.74 (d, 1H, ArH), 9.53 (s, 1H, N=CH). Analysis ($C_{11}H_{12}N_4O_3$) C, H, N.

*6-Amino-5-(4-bromobenzylidene)amino-1,3-dimethyluracil **2h***

Yield: 0.42 g (85%), m. p. 173 – 175°C (ethanol). ^{1}H-NMR: δ [ppm] = 3.17 (s, 3H, N_1-CH_3), 3.39 (s, 3H, N_3-CH_3), 7.39 (s, 2H, C_6-NH_2), 7.56 – 7.58 (d, 2H, ArH), 7.84 – 7.87 (d, 2H, ArH), 9.67 (s, 1H, N=CH). Analysis ($C_{13}H_{13}BrN_4O_2$) C, H, N.

*6-Amino-1,3-dimethyl-5-(4-nitrobenzylidene)aminouracil **2i***

Yield: 0.40 g (91%), m. p. > 300°C (DMF). ^{1}H-NMR: δ [ppm] = 3.18 (s, 3H, N_1-CH_3), 3.42 (s, 3H, N_3-CH_3), 7.58 (s, 2H, C_6-NH_2), 8.14 – 8.19 (dd, 4H, ArH), 9.79 (s, 1H, N=CH). Analysis ($C_{13}H_{13}N_5O_4$) C, H, N.

*N,N′-Bis-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimid-5-yl)ethylidinediamine **3***

To a solution of compound **1** (0.5 g, 2.42 mmol) in hot water (30 mL), ammonium hydroxide was added to pH 8. After cooling, glyoxal solution (1.21 mmol) was added with stirring for 20 min; the separated yellow product was filtered, washed with ether and crystallized.

Yield: 0.56 g, (64%), m. p.: 273 – 275°C (DMF/H_2O).^{1}H-NMR: δ [ppm] = 3.14 (s, 6H, $2CH_3$), 3.36 (s, 6H, $2CH_3$), 7.36 (s, 4H, $2 \times NH_2$, exch.), 9.34 (s, 2H, 2CH). Analysis ($C_{14}H_{18}N_8O_4$) C, H, N.

*General procedure for the preparation of compounds **4a – f***

To a solution of **2a – f** (1.2 mmol) in DMF (5 mL), triethyl orthoformate (1.5 mmol) was added and the mixture was heated under reflux for 10 h. After cooling, the reaction mixture was poured on to cold water and the formed product was filtered, washed with cold ethanol (90%), and then crystallized.

*1,3-Dimethyl-2,4-dioxo-6-(4-methoxyphenyl)-1,2,3,4-tetrahydropteridine **4a***

Yield: 0.11 g (31%), m. p. 219 – 220°C as reported in [13] (**4a** was separated by column chromatography of the obtained mixture of **4a** and **5a** on silica gel using toluene : ethyl acetate, 1 : 1). ^{1}H-NMR: δ [ppm] = 3.38 (s, 3H, N_1-CH_3), 3.58 (s, 3H, N_3-CH_3), 3.84 (s, 3H, OCH_3), 7.09 – 7.19 (d, 2H, ArH), 8.14-8.19 (d, 2H, ArH), 9.38 (s,1H, C_7-H). Analysis ($C_{15}H_{14}N_4O_3$) C, H, N.

*1,3-Dimethyl-2,4-dioxo-6-(phenyl)-1,2,3,4-tetrahydropteridine **4b***

Yield: 0.25 g (80%), m. p. > 300°C as reported in [13] (ethanol). ^{1}H-NMR: δ [ppm] = 3.34 (s, 3H, N_1-CH_3), 3.57 (s, 3H, N_3-CH_3), 7.47 – 7.58

(m, 3H, ArH), 8.10 – 8.17 (m, 2H, ArH), 9.37 (s, 1H, C_7-H). Analysis ($C_{14}H_{12}N_4O_2$) C, H, N.

1,3-Dimethyl-2,4-dioxo-6-(2-pyridyl)-1,2,3,4-tetrahydropteridine ***4c***

Yield: 0.16 g (50%) m. p. 270 – 271°C (purified by column chromatography on silica gel using chloroform : methanol, 9 1). ^{1}H-NMR: δ [ppm] = 3.35 (s, 3H, N_1-CH_3), 3.58 (s, 3H, N_3-CH_3), 7.49 – 7.54 (t, 1H, ArH), 8.02 – 8.05 (t, 1H, ArH), 8.29 – 8.32 (d, 1H, ArH), 8.72 – 8.74 (d, 1H, ArH), 9.58 (s, 1H, C_7-H). Analysis ($C_{13}H_{11}N_5O_2$) C, H, N.

1,3-Dimethyl-2,4-dioxo-6-(4-pyridyl)-1,2,3,4-tetrahydropteridine ***4d***

Yield: 0.224 g (70%), m. p. 235 – 236°C (purified by column chromatography on silica gel using $CHCl_3$: CH_3OH, 9 : 1). ^{1}H-NMR: δ [ppm] = 3.34 (s, 3H, N_1-CH_3), 3.58 (s, 3H, N_3-CH_3), 8.09 – 8.14 (d, 2H, ArH), 8.74 – 8.82 (d, 2H, ArH), 9.49 (s,1H, C_7-H). Analysis ($C_{13}H_{11}N_5O_2$) C, H, N.

6-Acetyl-1,3-dimethyl-2,4-dioxo- 1,2,3,4-tetrahydropteridine ***4e***

Yield: 0.16 g (57%), m. p. 192 – 194°C (ethanol); ^{1}H-NMR: δ [ppm] = 2.6 (s, 3H, CH_3), 3.33 (s, 3H, N_1-CH_3), 3.56 (s, 3H, N_3-CH_3), 9.18 (s, 1H, C_7-H). Analysis ($C_{10}H_{10}N_4O_3$) C, H, N.

1,3-Dimethyl-2,4-dioxo-6-ethoxycarbonyl-1,2,3,4-tetrahydropteridine ***4f***

Yield: 0.14 g (44%), m. p. 115 – 117°C (ethanol).^{1}H-NMR: δ [ppm] = 1.32 – 1.38 (t, 3H, CH_3), 3.33 (s, 3H, N_1-CH_3), 3.56 (s, 3H, N_3-CH_3), 4.40 – 4.42 (q, 2H, CH_2), 9.26 (s, 1H, C_7-H). Analysis ($C_{11}H_{12}N_4O_4$) C, H, N.

Preparation of compounds ***5a, b, c***

A mixture of **2b** or 2 g (1.2 mmol) and 1.0 mL of triethyl orthoacetate or triethyl orthobenzoate in DMF (5 mL) was heated under reflux for 10 h. After cooling, the separated product was filtered, washed with ether, and crystallized.

1,3-Dimethyl-8-(4-methoxyphenyl)-9(H)-xanthine ***5a***

Yield: 0.19 g (55%), m. p. >300°C (**5a** was separated by column chromatography of the previously obtained mixture of **4a** and **5a** on silica gel using toluene : ethyl acetate, 1 : 1). ^{1}H-NMR: δ [ppm] = 3.23 (s, 3H, N_1-CH_3), 3.46 (s, 3H, N_3-CH_3), 3.80 (s, 3H, OCH_3), 7.01 – 7.05 (d, 2H, ArH), 8.03 – 8.06 (d, 2H, ArH), 13.56 (s, 1H, C_9-H). Analysis ($C_{14}H_{14}N_4O_3$) C, H, N.

1,3-Dimethyl-8-(phenyl)-9(H)-xanthine ***5b***

Yield: 0.19 g (61%), m. p. >300°C (DMF/H_2O). ^{1}H-NMR: δ [ppm] = 3.22 (s, 3H, N_1-CH_3), 3.48 (s, 3H, N_3-CH_3), 7.41 – 7.56 (m, 3H, ArH), 8.12 – 8.19 (m, 2H, ArH), 13.83 (s, 1H, C_9-H). Analysis ($C_{13}H_{12}N_4O_2$) C, H, N.

1,3-Dimethyl-8-(2-furyl)-9(H)-xanthine ***5c***

Yield: 0.17 g (57%), m. p.: >300°C (purified by column chromatography on silica gel using $CHCl_3$: CH_3OH system, 9 : 1).^{1}H-NMR: δ [ppm] = 3.24 (s, 3H, N_1-CH_3), 3.45 (s, 3H, N_3-CH_3), 6.68 – 6.69 (t, 1H, ArH), 7.20 – 7.22 (d, 1H, ArH), 7.90 (s, 1H, ArH), 13.92 (s, 1H, C_9-H, exch.). Analysis ($C_{11}H_{10}N_4O$) C, H, N.

Biological investigation

Nucleic acids preparation

Plasmids DNA and total RNA were extracted and purified by the alkali method [14 – 16]. The cells of *E. coli*, isolated from clinical specimen and identified by the API20E system (BioMereuex, France) were grown for 16 – 18 h., then harvested by centrifugation at 14 000 rpm/min. The cells were resuspended in solution I (Glucose 50 mM, Tris-HCl, pH 8, 5 mM and EDTA, pH 8, 10 mM), then a double volume from solution II (NaOH, 0.2M and SDS, 1% w/v) was added. The cells were degraded and the solution became clear after keeping it in ice for 1 h. Proteins and chromosomal DNA were precipitated after addition of solution III (K-acetate, 5 M) in 1.5 volumes of solution I. The mixture was kept in crushed ice for 1 – 2 h, and then was centrifuged at 14 000 rpm for 15 min at 4°C. The supernatant containing plasmids DNA and total RNA was extracted once with phenol/chloroform and another time with chloroform. The supernatant was transferred to a clean sterile Eppendorf tube containing about 1 mL of absolute ethanol cooled to – 20°C, and kept in ice for 1 h. The plasmid DNA and RNA were precipitated and collected by short centrifugation at 14 000 rpm for 2 min at 4°C. The precipitate was washed once with 70% ethanol cooled to -20°C, then air-dried and dissolved in TE buffer (Tris-HCl, pH 8, 10 mM, and EDTA, 1 mM). The concentration of nucleic acids was measured spectrophotometrically at λ_{260}.

Agarose gel preparation

0.8 g ultra agarose was boiled in Tris-Acetate-EDTA (TAE) buffer and then cooled to ~60°C before pouring to the gel track.

Nucleic acids binding assay

The test compounds were dissolved in DMSO about 10 μg/10 μL. Nucleic acids were mixed with the test compounds 10 μg/10 μL and kept at room temperature for 15 min. The mixture was mixed with gel-loading buffer and then electrophoresed in the agarose gel (0.8% w/v) at 80 V for 1.5 h. Similarly, a mixture of nucleic acids and acyclovir, 10 μg/10 μL, was used as a positive control for affinity, binding, and fragmentation. DMSO plus nucleic acids were used as a negative control. Ethidium bromide (0.1 mg/mL) solution was used to stain the nucleic acid (DNA and RNA) bands in the gel to be visualized on UV transilluminator. The gel was photographed by polarized camera (Fig. 1).

Antiviral screening

Materials and methods

Stock solutions (1 mg/mL) of the chosen compounds were made in DMSO and were subsequently diluted in the appropriate culture media. The final DMSO concentration was maximum 0.1% and it was shown that this concentration had no effect on the cell cultures [22]. Therefore, 0.1% DMSO was also added to all no-drug control samples.

Peste des petits ruminant virus (PPRV)

PPR virus strain was used at its 20th passage on Vero cells [18]. The virus titer was 10^6 $TCID_{50}$/75 μL. Cell culture: Vero cells were isolated from kidneys of African green monkeys and were used for PPRV propagation [19]. Minimum Essential Medium (MEM) with Hank's salts [23] was used for cell culture preparation and cell passages. It was supplemented with 10% fetal calf serum.

The medium contained 50 μg of streptomycin and 100 units of penicillin/mL of growth medium.

Cytotoxicity assay

It was conducted to determine the highest dose of each compound that could be added to Vero cells without causing appreciable cytotoxicity [20]. Microtiter wells containing Vero cells (100 μL/well) were inoculated with 25 μL of graded concentrations of ribavirin and the test compounds in Hank's balanced salt solution (HBSS, Difco). Three replicates per concentration were used. Cell culture control wells were treated with 25 μL of HBSS alone. The plates were incubated at 37°C. After 72 h, the fluid was removed from the wells and the cells were stained with 0.15% crystal violet in 2% ethanol (100 μL/well) for 10 min. The stain was removed and the plates were rinsed gently with water and air-dried in a laminar-flow hood. The absorbance at 595 nm was recorded. The highest concentration of each compound which did not differ significantly in absorbance from the control wells was the concentration tested in the subsequent antiviral assay. For each compound, the concentration that reduced the absorbance reading to 50% of the control-well level (CD_{50}) was calculated by curve-fit analysis using commercially available computer graphics software.

Cytopathic effect reduction assays

These were performed in 96-well microtiter plates [20]. PPR virus prepared in HBSS medium (75 μL) were added to the wells containing Vero cells (100 μL/well) and the highest noncytotoxic concentration of each compound or ribavirin (25 μL/well). The resulting virus titers were compared with virus control titrations made in parallel. The test compounds were added with the infection and 1 h. after infection in the highest non-cytotoxic concentration. Three replicates per concentration were used. The plates were incubated at 37°C for 72 to 96 h. End point titers (log10 $TCID_{50}$) were determined by evaluating the cytopathic effect and were calculated by the accumulative method of Reed and Muench [21] and then presented as the% reduction in the cytopathic effects (Table 1).

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