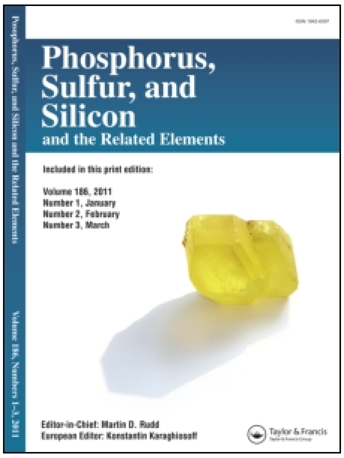

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Synthesis, Reactions, and Antiviral Activity of 1-(1*H*-Pyrazolo[3,4-*b*]pyridin-5-yl)ethanone and Pyrido[2',3':3,4]pyrazolo[5,1-*c*][1,2,4]triazine Derivatives

Fawzy A. Attaby[a]; A. H. H. Elghandour[a]; M. A. Ali[b]; Yasser M. Ibrahem[c]
[a] Chemistry Department, Cairo University, Giza, Egypt [b] Virology Lab., National Research Center, Dokki, Egypt [c] Chemistry Department, Cairo University, Cairo, Egypt

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Synthesis, Reactions, and Antiviral Activity of 1-(1*H*-Pyrazolo[3,4-*b*]pyridin-5-yl)ethanone and Pyrido[2′,3′:3,4]pyrazolo[5,1-*c*][1,2,4]triazine Derivatives

Fawzy A. Attaby
A. H. H. Elghandour
Chemistry Department, Cairo University, Giza, Egypt

M. A. Ali
Virology Lab., National Research Center, Dokki, Egypt

Yasser M. Ibrahem
Chemistry Department, Cairo University, Cairo, Egypt

1-(3-Amino-6-methyl-4-pyridin-3-yl-1H-pyrazolo[3,4-b]pyridin-5-yl)ethanone (**3**) *was obtained in very pure state and used as a good starting material for the present study. It diazotized to give the corresponding diazonium salt* **9** *and also reacted with phenyl isothiocyanate to give the corresponding thiourea derivative* **4**. *Compound* **4** *was used for the preparation of thiazole derivatives* **5–8** *via the reaction with active halogen-containing compounds. On the other hand, compound* **9** *coupled with several active* $-CH_2-$ *containing compounds to afford the corresponding triazine derivatives* **10–17**. *Considering the data from IR,* ^1H *NMR, mass spectra, and elemental analyses, the chemical structures of the newly synthesized heterocyclic compounds were elucidated. Cytotoxicity, anti-HSV1, and anti-HAV-MBB activity were evaluated for the newly synthesized heterocyclic compounds.*

Keywords N-phenylthiourea; 1H-pyrazolo[3,4-b]pyridin-5-yl)ethanone; 1,3-thiazolidin-4-one; 2-cyanoethanethioamide; pyrido[2′,3′:3,4]pyrazolo[5,1-c] [1,2,4]triazine

INTRODUCTION

In previous papers,[1–17] the synthesis of pyrazolopyridine and pyridopyrazolotriazine derivatives were described. As a continuation of our research on the synthesis of these ring systems and owing to the reported biological activities of pyrazolopyridine[18,19] and thiazoles,[20–22]

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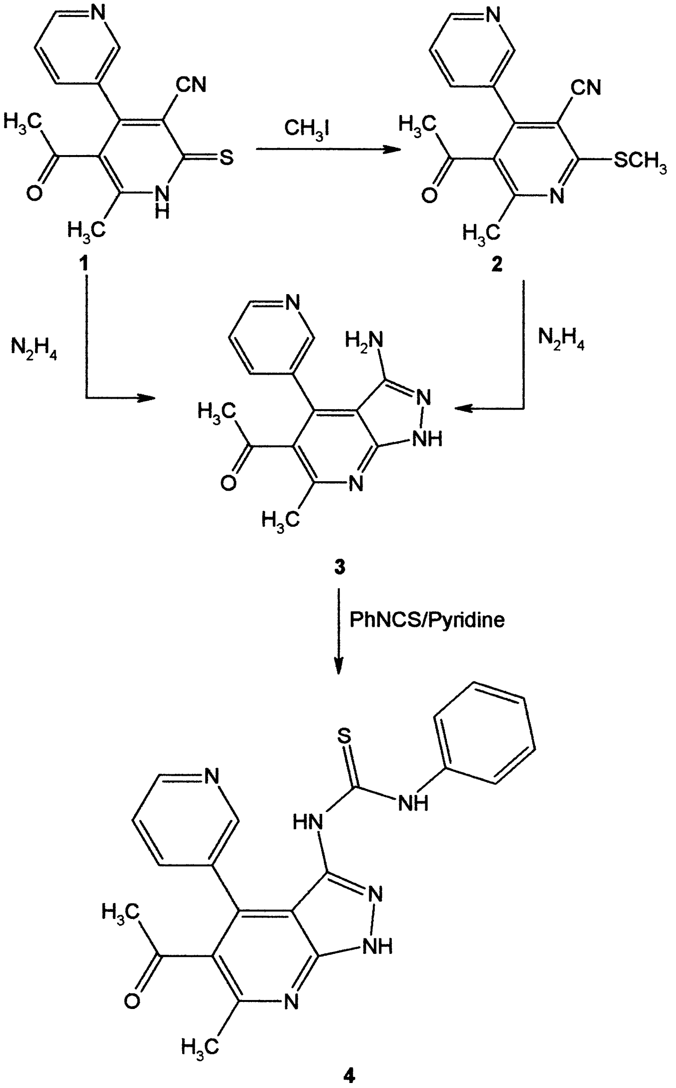
Address correspondence to Fawzy A. Attaby, Cairo University, Chemistry Department, Faculty of Science, Giza, Egypt. E-mail: fattaby@hotmail.com as well as that of triazines,[23–26] we were interested to synthesize several derivatives of these ring systems that are required for several chemical transformations as well as for our medicinal chemistry program.

RESULTS AND DISCUSSION

It was found that 5′-acetyl-6′-methyl-2′-(methylthio)-3,4′-bipyridine-3′-carbonitrile (**2**) reacted with hydrazine hydrate in ethanol to give the sulfur-free 1-(3-Amino-6-methyl-4-pyridin-3-yl-1H-pyrazolo-[3,4-b]pyridin-5-yl)ethanone (**3**). The chemical structure of **3** was elucidated based on the IR, ^{1}H NMR, and elemental analysis (cf. Experimental section). Moreover, its mass spectrum gave m/z = 267, which corresponded to the molecular weight of a molecular formula $C_{14}H_{13}N_5O$ of the assigned structure. A further elucidation of structure **3** arose from its preparation from the reaction of 5′-acetyl-6′-methyl-2′-thioxo-1′,2′-dihydro-3,4′bipyridine-3′-carbonitrile (**1**) with hydrazine hydrate. It is remarkable that the product of the two pathways was identical in all physical and chemical properties (cf. Exp. section and Scheme 1).

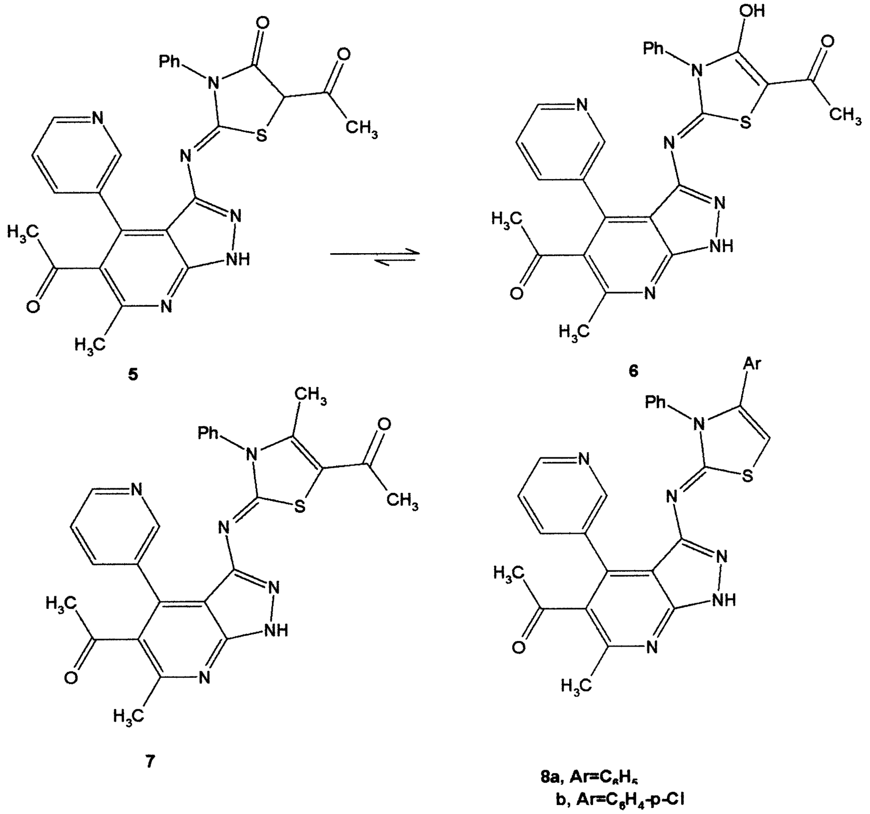
The synthetic potentiality of **3** was investigated through its reaction with phenyl isothiocyanate in pyridine to give the corresponding N-(5-acetyl-6-methyl-4-pyridin-3-yl-1H-pyrazolo[3,4-b]pyridin-3-yl)-N-phenyl-thiourea (**4**). The IR, ^{1}H NMR, and elemental analysis of this reaction product were found to be in a good agreement with the assigned structure (cf. Exp. section and Scheme 1). Moreover, the mass spectrum of **4** gave m/z = 402, which corresponded to the molecular weight of a molecular formula $C_{21}H_{18}N_6OS$ of the assigned structure.

Compound **4** reacted with ethyl 2-chloro-3-oxobutanoate, 3-chloropentan-2,4-dione, 2-bromo-1-phenylethanone, and 2-bromo-1-(4′-chlorophenyl)-ethanone in pyridine to give 1,3-thiazolidin-4-one derivative **5**, which tautomerized to the more stable isomer1-[(5-acetyl-3-phenyl-4-hydroxy-1,3-thiazol-2(3*H*)-ylidene)amino]-6-methyl-4-pyridin-3-yl-1*H*-pyrazolo[3,4-*b*]pyridin-5-yl)ethanone (**6**), 4-(3-pyridyl)-5-acetyl-6-methyl-N-[4-methyl-3-phenyl-5-acetyl-1,3-thiazol-2(2H)ylidine]-1H-pyrazolo-[3,4-b]pyridin-3-amine (**7**), and 1-([3,4-diaryl-1,3-thiazol-2-ylidine]amino-6-methyl-4-pyridin-3-yl-1H-pyrazolo-[3,4-b]-pyridin-5-yl)ethanone **8a**,**b**, respectively. The chemical structures of compounds **6–8** were elucidated by considering the data of IR, 1H NMR, and elemental analyses (cf. Experimental section). Moreover, the mass spectra of **6**, **7**, and **8a**,**b** gave m/z = 442, 482, 502, and 537, which corresponded to the molecular weights of the molecular formulas $C_{23}H_{18}N_8O_2S$, $C_{26}H_{22}N_6O_2S$, $C_{29}H_{22}N_6O_2S$, and $C_{29}H_{21}N_6OS$, of the assigned structures, respectively (cf. Exp. section and Scheme 2).



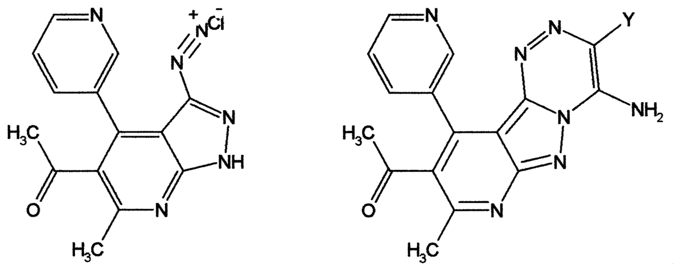
SCHEME 1

The chemical reactivity of **3** was further investigated via its reaction with nitrous acid to give the corresponding 3-diazotized aminopyrazolo[3,4-b]pyridine derivative **9**, which was used for the preparation of triazine derivatives **10–17** via its coupling with several active hydrogen-containing compounds. It is remarkable to report that all trials to isolate **9** as a solid product failed. Thus, the treatment of **9**



SCHEME 2

with malononitrile, ethyl cyanoacetate, and 2-cyanoethanethioamide in cold ethanolic sodium acetate gave reaction products, which corresponded to addition of equimolecular amounts of the reactants with dehydrochlorination. The IR spectra of these reaction products showed the bands of NH_2 that were detected also in the ^{1}H NMR spectra. Moreover, the IR spectra of these reaction products showed the CN function, CO ester, and $CSNH_2$ respectively (cf. Experimental section). By considering the data of IR, ^{1}H NMR, and elemental analyses, these reaction products could be formulated as 9-acetyl-4-amino-8-methyl-10-pyridin-3-ylpyrido[2′,3′:3,4]pyrazolo[5,1-c]-[1,2,4]triazine-3-carbonitrile (**10**), ethyl9-acetyl-4-amino-8-methyl-10-pyridin-3-ylpyrido[2′,3′:3,4]pyrazolo[5,1-c][1,2,4]-triazine-3-carboxylate (**11**), and 1-(4-amino-3-thiocarboxamido-8-methyl-10-pyridin-3-yl pyrido-[2’,3’:3,4]-pyrazolo[5,1-c][1,2,4]triazin-9-yl)ethanone (**12**), respectively. The



9

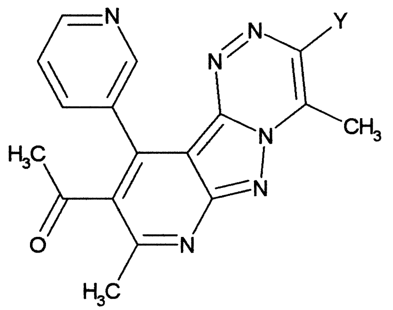
10 (Y = CN), 11 (Y = COOEt) and 12 (Y = CSNH$_2$)

SCHEME 3

mass spectra of **10**, **11**, and **12** gave m/z = 346, 393, and 389, which corresponded to the molecular weights of the molecular formulas $C_{17}H_{14}N_8O$, $C_{19}H_{19}N_7O_3$, and $C_{17}H_{16}N_8OS$ of the assigned structure, respectively (Scheme 3).

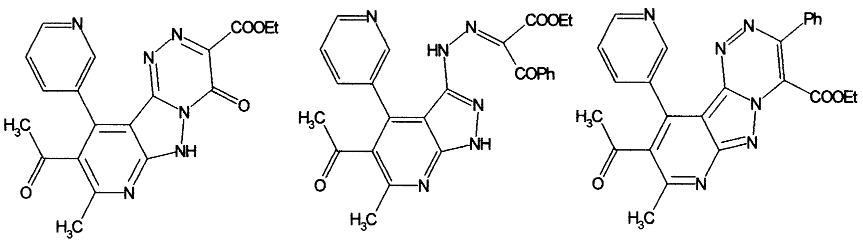
In a similar reaction, **9** reacted with ethyl 3-oxobutanoate and/or pentan-2,4-dione in cold ethanolic sodium acetate to afford the corresponding triazine derivatives **13** and **14** whose structures were elucidated from IR, ^{1}H NMR, and elemental analyses (cf. Experimental section). Moreover, their mass spectra gave m/z = 392 and 362, which corresponded to the molecular weights of the molecular formulae $C_{20}H_{20}N_6O_3$ and $C_{19}H_{18}N_6O_2$ of the assigned structures. A further confirmation of the **10**, **11**, **12**, **13**, and **14** structures obtained from the reactions between **9** and malononitrile, ethyl cyanoacetate, 2-cyanoethanethioamide, ethyl 3-oxobutanoate, and/or pentan-2,4-dione in boiling ethanol containing catalytic amounts of triethylamine. It is important to refer here that the reaction products obtained by the two pathways were identical in all physical and chemical properties (cf. Exp. section and Scheme 4).

Compound **9** reacted with diethyl malonate in cold ethanolic sodium acetate to give a reaction product **15** via dehydrochlorination, followed by cyclization through ethanol elimination. The structure of **15** was established from analysis of IR, ^{1}H NMR, and elemental analyses (cf. Experimental section) Moreover, its mass spectrum gave m/z = 394, which corresponded to the molecular weight of a molecular formula of the assigned structure. In contrast to the behavior of **9** in the previously mentioned reactions, **9** reacted with ethyl 3-oxo-3-phenylpropanoate under the same experimental conditions to give the open-chain structure **16**, which was obtained through the dehydrochlorination. Compound **16** underwent cyclization by treatment with triethylamine in boiling ethanol to give the corresponded triazine derivative **17** whose structure was



13 (Y = COOEt), **14** (Y = $COCH_3$)

SCHEME 4



15 **16** **17**

SCHEME 5

elucidated from analysis of IR, ^{1}H NMR, and elemental analyses (cf. Exp. section and Scheme 5).

Further confirmation of both **15** and **17** structures was given through their preparation authentically by boiling **9** with diethyl malonate and ethyl 3-oxo-3-phenylpropanoate, respectively, in ethanol containing a catalytic amount of triethylamine (cf. Experimental section).

Biological Evaluation

Cytotoxicity Assay

As shown in Table (I), compounds **3**, **10**, **11**, **12**, **13**, **14**, **15**, and **17** were safe for cell culture inoculation than were the other compounds, which show CPE (cytopathic effect) at 15 μg. Accordingly, the safe doses were selected and used for an antivirus bioassay.

TABLE I Cytotoxicity Assay for the Synthetic Compound in a Vero Cell Line

	Cytotoxicity grade of the tested materials							
Ser. no.	5 μg	10 μg	15 μg	20 μg	25 μg	30 μg	35 μg	40 μg
3	—	—	—	—	—	—	—	—
4	+2	+4	+4	+4	+4	+4	+4	+4
6	+1	+4	+4	+4	+4	+4	+4	+4
7	+2	+4	+4	+4	+4	+4	+4	+4
8a	+4	+4	+4	+4	+4	+4	+4	+4
8b	+1	+3	+4	+4	+4	+4	+4	+4
10	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	+1	+1
12	—	—	+1	+1	+1	+2	+3	+4
13	—	—	+1	+1	+1	+2	+3	+4
14	—	—	—	+1	+2	+3	+4	+4
15	—	—	—	+1	+1	+2	+2	+2
16	—	—	+1	+3	+4	+4	+4	+4
17	—	—	—	—	—	+1	+1	+1

Cytotoxicity grades are divided into four grads: +1 = 25% of the cell monolyer showed CPE; +2 = 50% of the cell monolayer showed CPE; +3 = 75% of the cell monolayer showed CPE; and +4 = 100% of the cell monolayer showed CPE.

Antivirus Bioassay

Screening for anti-HSV1 activity. Plaque reduction assay showed that compounds **3**, **4**, **6**, and **7** were promising as anti-HSV1 compounds at a dose of 15 μg, and the percentage of virus reduction increased at 25 μg. Compound **12** only gave virus reduction by 55% at a dose of 15 μg, and compound **8b** only exhibited virus reduction at a dose of 25 μg. All other compounds either had very week activity or no activity at all (cf. Figure 1).

Screening for Anti-HAV-MBB Strain Activity

All compounds were tested for anti-HAV activity in HepG2 cells by plaque reduction assay. The results showed that compounds **4**, **6**, and **7** exhibited good percentages of virus reduction at both 15 μg and 25 μg doses, while compound **8a** exhibited a 53% virus reduction at a dose of 25 μg. Compound **8b** showed moderate activity at both 15 μg and 25 μg doses. The other newly synthesized heterocyclic compounds exhibited no activity against HAV (cf. Figure 2).

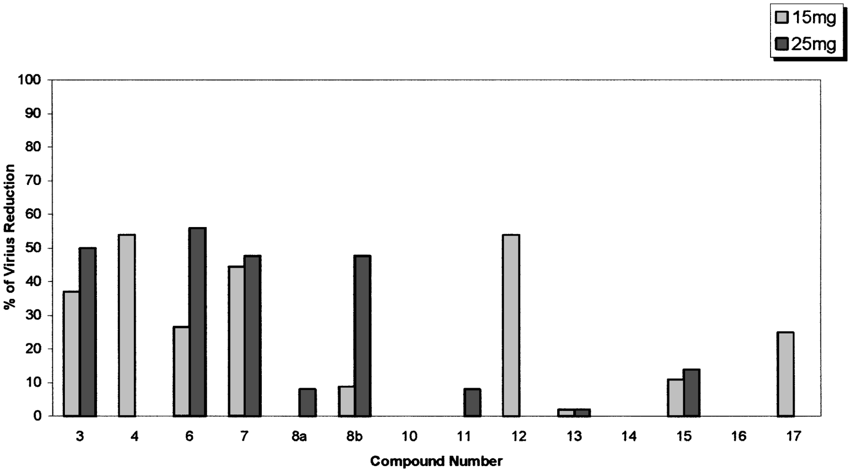


FIGURE 1 Anti-HSV1 bioassay of synthetic compounds in a Vero cell line.

EXPERIMENTAL

All melting points were uncorrected. IR (KBr discs) spectra were recorded on a Shimadzu FTIR-8201PC spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian Mercury 300 MHz and Varian Gemini 200 MHz. spectrometers using TMS as an internal standard and $CDCl_3$, DMSO-d_6, and $(CD_3)_2CO$ as solvents. Chemical shifts were expressed

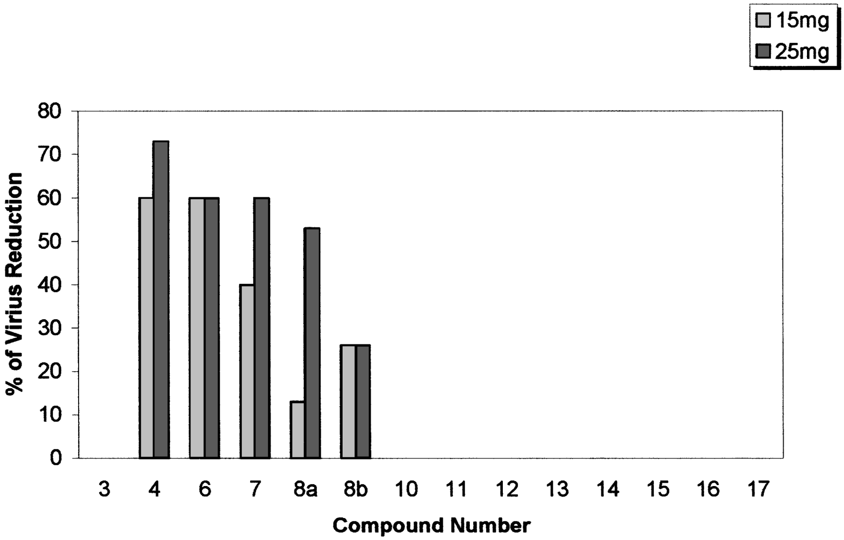


FIGURE 2 Anti-HAV bioassay of synthetic compounds in HepG2 cells.

as δ (ppm) units. Mass spectra were recorded on Shimadzu GCMS-QP1000EX using an inlet type at 70 eV. The Microanalytical Center of Cairo University performed the microanalyses.

Synthesis of 3

A mixture of **2** or **1** (0.01 mole of each) and hydrazine hydrate (20–25 mL) was heated under reflux for 8–10 h. The product formed after cooling was filtered off, washed with cold ethanol, and crystallized from ethanol to give **3**.

1-(3-Amino-6-methyl-4-pyridin-3-yl-1H-pyrazolo[3,4-b]pyridin-5-yl)-ethanone (3)

Crystallized from ethanol as yellow crystals (81%); m.p. 284–286°C; IR (cm^{-1}): 3450, 3267, 3179 (NH_2, NH), 3043 (pyridine-CH), 2982 (sat. CH) and 1705 (acetyl-CO); ^{1}H-NMR (δ): 1.3 (s, 3H, CH_3), 2.5 (s, 3H, CH_3CO), 3.9 (s, br., 2H, NH_2), 5.8(s, br., 1H, NH) and 7.3–8.3 (m, 4H, Pyridine H's); Anal. for $C_{14}H_{13}N_5O$ (267): Calcd./Found: C, 62.91/63.1%; H, 4.90/5.0%; N, 26.20/26.4%.

Synthesis of 4

A solution of **3** (0.01 mole) in pyridine (35 mL) was treated with phenyl isothiocyanate (0.01 mole). The reaction mixture was heated under reflux for 5 h, then cooled, poured onto ice-cold water, and acidified with dilute HCl. The solid product so formed was filtered off, washed with water, and crystallized from ethanol to give **4**.

1-(5-Acetyl-6-methyl-4-(pyridin-3-yl)-1H-pyrazolo[3,4-b]-pyridin-3-yl)-3-phenylthiourea (4)

Crystallized from ethanol as white crystals, (77%), m.p. 206–208°C; IR (cm^{-1}): 3382, 3277, 3205 (three NH), 3049 (pyridine-CH), 2986 (sat. CH) and 1701 (acetyl-CO); ^{1}H-NMR (δ): 1.2 (s, 3H, CH_3), 2.4 (s, 3H, CH_3CO), 5.8–6.3 (m, 3H, three NH) and 7.5–8.7 (m, 9H, ArH's and Pyridine H's); Anal. for $C_{21}H_{18}N_6OS$ (402): Calcd./Found: C, 62.67/62.4%; H, 4.51/4.5%; N, 20.88/21.1%; S, 7.97/8.1%.

Synthesis of 6

A solution of **4** (0.01 mole) in ethanol (50 mL) in the presence of sodium acetate (1 gm) was heated under reflux with ethyl 2-chloro-3-oxobutanoate (0.01 mole) for 5 h. After cooling, the solid so formed was filtered off, washed with water, and crystallized with ethanol to give **6**.

1-[(5-Acetyl-3-phenyl-4-hydroxy-1,3-thiazol-2(3H)-ylidene)amino]-6-methyl-4-pyridin-3-yl-1H-pyrazolo[3,4-b]pyridin-5-yl)ethanone(6)

Crystallized from ethanol as pale yellow crystals (69%) m.p. 248–250°C; IR (cm^{-1}): 3452, (OH), 3175 (NH), 3050 (aromatic-CH), 2973 (sat. CH) and 1698 (acetyl-CO); ^{1}H-NMR (δ): 1.1 (s, 3H, CH_3), 2.5 (s, 6H, two $\underline{CH_3}$CO), 5.1 (s, 1H, OH) 6.3 (s, br., 1H, NH) and 7.2–8.4 (m, $\overline{9H,}$ ArH's and Pyridine H's); Anal. for $C_{25}H_{20}N_6O_3S$ (484): Calcd./Found: C, 61.97/62.1%; H, 4.16/4.2%; N, 17.34/17.1%; S, 6.62/6.7%.

Synthesis of 7

A solution of **4** (0.01 mole) in ethanol (50 mL) in the presence of sodium acetate (1 gm) was heated under reflux with 3-chloropentan-2,4-dione (0.01 mole) for 5 h. After cooling, the solid so formed was filtered off, washed with water, and crystallized with ethanol to give **7**.

4-(3-Pyridyl)-5-acetyl-6-methyl-N-[4-methyl-3-phenyl-5-acetyl-1,3-thiazol-2(2H)-ylidine]-1H-pyrazolo-[3,4-b]pyridin-3-amine (7)

Crystallized from ethanol as yellow crystals (72%) m.p. 264–266°C; IR (cm^{-1}): 3135 (NH), 3061 (aromatic-CH), 2879 (sat. CH) and 1707 (acetyl-CO); ^{1}H-NMR (δ): 1.3 (s, 6H, two CH_3), 2.5 (s, 6H, two CH_3CO), 6.2 (s, 1H, NH) and 7.3–8.5 (m, 9H, ArH's and Pyridine H's); Anal. for $C_{26}H_{22}N_6O_2S$ (482): Calcd./Found: C, 64.71/64.5%; H, 4.6/4.5%; N, 17.42/17.5%; S, 6.65/6.7%.

Synthesis of 8a,b

A solution of **4** (0.01 mole) in ethanol (50 mL) in the presence of sodium acetate (1 gm) was heated under reflux with 2-bromo-1-phenylethanone or 2-bromo-1-(4′-chlorophenyl)ethanone (0.01 mole) for 5 h. After cooling, the solids so formed were filtered off, washed with water, and crystallized with ethanol to give **8ab**, respectively.

1-([3,4-Diphenyl-1,3-thiazol-2-ylidine]amino-6-methyl-4-pyridin-3-yl-1H-pyrazolo[3,4-b]pyridin-5-yl)ethanone (8a)

Crystallized from ethanol asyellow crystals (61%) m.p. 146–148°C; IR (cm^{-1}): 3125 (NH), 3055 (aromatic-CH), 2883 (sat. CH) and 1689 (acetyl-CO); ^{1}H-NMR (δ): 1.2 (s, 3H, CH_3), 2.3 (s, 3H, CH_3CO), 5.1 (s, 1H, thiazole H-5), 5.9 (s, 1H, NH) and 7.2–8.4 (m, 14H, ArH's and Pyridine H's); Anal. for $C_{29}H_{22}N_6OS$ (502): Calcd./Found: C, 69.30/69.5%; H, 4.41/4.5%; N, 16.72/16.8%; S, 6.38/6.4%.

1-([3-Phenyl-4-(4-chlorophenyl)-1,3-thiazol-2-ylidine] amino-6-methyl-4-pyridin-3-yl-1H-pyrazolo-[3,4-b]pyridin-5-yl)ethanone (8b)

Crystallized from ethanol as yellowish-white crystals (71%) m.p. 172–174°C; IR (cm^{-1}): 3139 (NH), 3063 (aromatic-CH), 2878 (sat. CH) and 1695 (ketone-CO); ^{1}H-NMR (δ): 1.1 (s, 3H, CH_3), 2.5 (s, 3H, CH_3CO), 5.4 (s, 1H, thiazole H-5, 6.1 (s, 1H, NH) and 7.2–8.3 (m, 13H, ArH's and Pyridine H's); Anal. for $C_{29}H_{21}N_6OSCl$ (502): Calcd./Found: C, 64.86/65.0%; H, 3.94/4.1%; N, 15.65/15.7%; S, 5.97/6.1%; Cl, 6.60/6.5%.

Synthesis of 10–16: General Procedure

A solution of **9** (0.01 mole) in ethanol (50 mL) in the presence of sodium acetate (1 g) was treated with either malonitrile, ethyl cyanoacetate, 2-cyanoethanethioamide, ethyl 3-oxobutanoate, pentan-2,4-dione, ethyl 3-oxo-3-phenylpropanoate, or diethyl malonate (0.01 mole of each) respectively. The reaction mixture was stirred in an ice-cold bath for 1 h. The solid product obtained in each case was filtered off, washed with water, and crystallized from ethanol as **10–16**, respectively.

9-Acetyl-4-amino-8-methyl-10-pyridin-3-ylpyrido-[2′,3′:3,4]pyrazolo[5,1-c][1,2,4]triazine-3-carbonitrile (10)

Crystallized from ethanol as pale brown crystals (61%) m.p. > 300°C; IR (cm^{-1}): 3339, 3211 (NH_2), 3063 (pyridine-CH), 2872 (sat. CH) and 1703 (acetyl-CO); ^{1}H-NMR (δ): 1.1 (s, 3H, CH_3), 2.3 (s, 3H, CH_3CO), 5.4 (s, br., 2H, NH_2) and 7.0–8.1 (m, 4H PyridineH's); Anal. for $C_{17}H_{12}N_8O$ (344): Calcd./Found: C, 59.5/59.6%; H, 3.51/3.5%; N, 32.54/32.5%.

9-Acetyl-4-amino-8-methyl-10-pyridin-3-ylpyrido-[2′,3′:3,4]pyrazolo[5,1-c][1,2,4]triazine-3-carboxylate (11)

Crystallized from ethanol as yellow crystals (69%) m.p. 250°C; IR (cm^{-1}): 3359, 3231 (NH_2), 3058 (pyridine-CH), 2878 (sat. CH) and 1711 (ester-CO), 1702 (acetyl-CO); ^{1}H-NMR (δ): 1.1 (s, 3H, CH_3), 1.5 (t, 3H, CH_3CH_2), 2.4 (s, 3H, CH_3CO), 4.2 (q, 2H, CH_3CH_2), 5.5 (s, br., 2H, NH_2) and 7.1–8.5 (m, 4H, PyridineH's); Anal. for $C_{19}H_{17}N_7O_3$ (391): Calcd./Found: C, 58.31/58.4%; H, 4.38/4.2%; N, 25.05/25.0%.

1-(4-Amino-3-thiocarboxamido-8-methyl-10-pyridin-3-yl pyrido[2′,3′:-3,4]pyrazolo[5,1-c][1,2,4]triazin-9-yl)ethanone (12)

Crystallized from ethanol as brown crystals (65%) m.p. > 300°C; IR (cm^{-1}): 3452, 3298, 3152 (two NH_2), 3038 (pyridine-CH), 2867 (sat. CH) and 1697 (acetyl-CO), ^{1}H-NMR (δ): 1.1 (s, 3H, CH_3), 2.2 (s, 3H, CH_3CO), 5.0–5.5 (m, 4H, two NH_2) and 7.0–8.2 (m, 4H, Pyridine H's); Anal. for $C_{17}H_{14}N_8OS$ (378): Calcd./Found: C, 53.96/54.0%; H, 3.73/3.8%; N, 29.61/29.5%; S, 8.47/8.5%.

Ethyl 9-Acetyl-4,8-dimethyl-10-pyridin-3-ylpyrido-[2′,3′:3,4]pyrazolo[5,1- c][1,2,4]triazine-3-carboxylate (13)

Crystallized from ethanol as yellowish-white crystals (65%) m.p. 180°C; IR (cm^{-1}): 3058 (pyridine-CH), 2864 (sat. CH) and 1701 (acetyl-CO), ^{1}H-NMR (δ): 1.0 (s, 6H, two CH_3), 1.5 (t, 3H, CH_3CH_2), 2.3 (s, 3H, CH_3CO), 4.2 (q, 2H, CH_3CH_2) and 7.0–8.4 (m, 4H, Pyridine H's); Anal. for $C_{20}H_{18}N_6O_3$ (390): Calcd./Found: C, 61.53/61.4%; H, 4.65/4.5%; N, 21.53/21.5%.

1-(3-Acetyl-4,8-dimethyl-10-pyridin-3-ylpyrido-[2′,3′:3,4]pyrazolo[5,1-c][1,2,4]triazin-9-yl)ethanone (14)

Crystallized from ethanol as yellowish-white crystals (61%) m.p. 195°C; IR (cm^{-1}): 3071 (pyridine-CH), 2868 (sat. CH) and 1705 (acetyl-CO), ^{1}H-NMR (δ): 1.1 (s, 6H, two CH_3), 2.4 (s, 6H, two CH_3CO) and 7.2–8.3 (m, 4H, Pyridine H's); Anal. for $C_{19}H_{16}N_6O_2$ (360): Calcd./Found: C, 63.32/63.4%; H, 4.48/4.5%; N, 23.32/23.5%.

Ethyl 9-Acetyl-8-methyl-4-oxo-10-pyridin-3-yl-3,4-dihydropyrido[2′,3′:3,4]pyrazolo[5,1-c][1,2,4]triazine-3-carboxylate (15)

As pale-yellow crystals (62%) m.p. > 300°C; IR (cm^{-1}): 3198 (NH), 3063 (pyridine-CH), 2851 (sat. CH) and 1713 (ester-CO), 1697 (acetyl-CO), 1662 (ring-CO); ^{1}H-NMR (δ): 1.1 (s, 3H, CH_3), 1.5 (t, 3H, CH_3CH_2), 2.4 (s, 3H, CH_3CO), 4.3 (q, 2H, CH_3CH_2) 5.4 (s, br., 1H, NH) and 6.9–8.2 (m, 4H, PyridineH's); Anal. for $C_{19}H_{16}N_6O_4$ (392): Calcd./Found: C, 58.16/58.4%; H, 4.11/4.2%; N, 21.42/21.5%.

Ethyl [(5-Acetyl-6-methyl-4-pyridin-3-yl-1H-pyrazolo-[3,4-b]pyri-din-3-yl)hydrazono]phenylacetate (16)

As white crystals (69%) m.p. 120°C; IR (cm^{-1}): 3188 (NH), 3059 (aromatic-CH), 2859 (sat. CH) and 1710 (ester-CO), 1698 (acetyl-CO); ^{1}H-NMR (δ): 1.0 (s, 3H, CH_3), 1.4 (t, 3H, CH_3CH_2), 2.5 (s, 3H, CH_3CO), 4.2 (q, 2H, CH_3CH_2) 5.6 (s, br., 2H, two NH) and 7.0–8.4 (m, 9H, ArH's and Pyridine H's); Anal. for $C_{25}H_{22}N_6O_4$ (470): Calcd./Found: C, 63.82/63.9%; H, 4.71/4.8%; N, 17.86/18.0%.

Synthesis of 17

A solution of **15** (0.01 mole) in ethanol (50 mL) in the presence of triethylamine (0.5 mL) was heated under reflux for 3 h. The solid obtained was filtered off and crystallized from ethanol as **17**.

Ethyl 9-Acetyl-8-methyl-3-pheny-10-pyridin-3-ylpyrido [2′,3′:3,4]pyrazolo[5,1-c][1,2,4]triazine-4-carboxylate(17)

As pale-yellow crystals (71%) m.p. 224–226°C; IR (cm^{-1}): 3057 (aromatic-CH), 2862 (sat. CH) and 1715 (ester-CO), 1702 (acetyl-CO); ^{1}H-NMR (δ): 1.1 (s, 3H, CH_3), 1.5 (t, 3H, CH_3CH_2), 2.3 (s, 3H, CH_3CO), 4.3 (q, 2H, CH_3CH_2) and 7.1–8.5 (m, 9H, ArH's and Pyridine H's); Anal. for $C_{25}H_{20}N_6O_3$ (452): Calcd./Found: C, 66.36/66.2%; H, 4.46/4.6%; N, 18.5718.6%.

Biological Evaluation

I: Two types of cell lines were used for propagation of Herpes simplex virus type 1 (HSV-1) and hepatitis A virus (HAV-MBB strain). These cell lines: African geen monkey kidney cells (Vero) and human hepatoma cell line (Hep G2).

Viruses

Two models of DNA and RNA viruses were used for the bioassay. These viruses were Herpes simplex virus type 1 (HSV-1) and Hepatitis A virus, MBB strain (HAV-MBB).

II: Media and Supplements

Cell Culture Medium

Minimum Essential Media (MEM with Hank's balanced salt solution, GIBCO-BRL) was prepared and sterilized by filtration through a 0.22-μm pore-size nitrocellulose membrane. The pH value was adjusted at 7.4 by sodium bicarbonate.

Foetal Bovine Serum (FBS)

(Sigma) FBS was inactivated at 56°C for 30 min and used at a 10% final concentration for growth medium and at 2% for maintenance medium.

Antibiotic-Antimycotic Mixture (GIBCO-BRL)

100 X antibiotic-antimycotic mixtures consisted of 10,000 U penicillin G sodium 10,000 μg streptomycin sulfate and 25 μg of amphotericin B.

III: Cell Dissociation Solution (Trypsin-Versene Mixture)

Phosphate Buffered Saline

(PBS, pH 7.5, 0.15 M.) The buffer was prepared at the following concentrations: NaCl (8.9 g/L); KCl (0.2 g/L); KH_2PO_4 (0.12 g/L); Na_2HPO_4 (0.91 g/L); Deionized H_2O (up to 1 L). Ingredients were mixed gently in the order previously shown, and the pH value was adjusted at 7.5. The buffer was sterilized by filtration through a 0.22 μm nitrocellulose membrane. The solution was used to wash the cell monolayer sheets and to prepare a cell dissociation solution as follows.

Trypsin 1:250 (Sigma)

1.5 gm of trypsin powder (1:250) was dissolved in 500 mL PBS and digested at 4°C overnight with stirring.

Versene Solution (0.04%)

Tetrasodium salt of ethylenediamine tetraacetic acid (EDTA) was dissolved in 500 mL of 0.15 M PBS (pH 7.5) to prepare a 2 mM solution (0.04 gm), which was mixed with an equal volume of trypsin solution. The pH value of the trypsin-versene mixture was adjusted to 8.4 by 7.5% sodium bicarbonate solution and sterilized by filtration through a 0.22-μm pore-size nitrocellulose membrane; the mixture was aliquoted and stored at – 20°C until used.

Methods

I: Preparation of Synthetic Compounds for Bioassay

Compounds were dissolved as 100 mg each in 1 mL of 10% DMSO in water. The final concentration was 100 μg/μL (stock solution). The dissolved stock solutions were sterilized by the addition of antibiotic-antimycotic mixture: 10,000 U penicillin G sodium or Gentamicin (50 μg/mL), 10,000 μg streptomycin sulfate, and 250 μg of amphotericin B. Sterility tests were carried out in nutrient agar.

II: Cell Culture

African green monkey kidney-derived cells (VERO) were used. The cells were propagated in Hanks' Minimum essential medium (MEM) and supplemented with 10% Foetal bovine serum and 1% antibiotic-antimycotic mixture. The pH was adjusted to 7.2–7.4 by 7.5% sodium bicarbonate solution. The mixture was sterilized by filtration through a 0.2-μm pore-size nitrocellulose membrane.

III: Viruses

Herpes Simplex virus type 1 was obtained from Environmental Virology Lab. Department of Water Pollution Res., National Research Center, Dokki, Egypt.

IV: Antiviral Assay

Cytotoxicity assay. Cytotoxicity was assayed for both dimethyl sulfoxide (DMSO) and the test compounds. Serial dilutions were prepared and inoculated on Vero and HepG2 cells grown in 96-well tissue culture plates. The maximum tolerated concentration for each compound was determined by both cell morphology and cell viability by straining with trypan blue dye.

Plaque reduction assay. A 6-well plate was cultivated with Vero cell culture (10^5cell/mL) and incubated for 2 days at 37°C. HSV-1 was diluted to give 10^4PFU/mL as a final concentration, mixed with the plant extract at the previous concentration, incubated overnight at 4°C. Growth medium was removed from the multiwell plate, and the virus-compound mixture was inoculated (100 μL/well). After 1 h of a contact time, the inoculum was aspirated, and 3 mL of MEM with 1% agar rose was overlaid the cell sheets. The plates were left to solidify and incubated at 37°C until the development of virus plaques. Cell sheets were fixed in 10% formaline solution for 2 h and stained with crystal violet stain. Control virus, and cells were treated identically without chemical compounds. Virus plaques were counted, and the percentage of reduction was calculated.

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