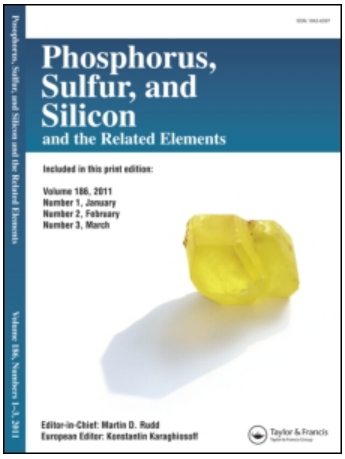

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Synthesis, Reactions, and Antiviral Activity of 5'-Acetyl-6'-methyl-2'-thioxo-1',2'-dihydro-3,4'-bipyridine-3'-carbonitrile

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Synthesis, Reactions, and Antiviral Activity of 5′-Acetyl-6′-methyl-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′-carbonitrile

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Bipyridine-3′0-carbonitrile derivatives ***5*** *reacted with several halogen containing reagents, e.g., 1-chloroacetone, 3-chloropentan-2,4-dione, ethyl chloroacetate, ethyl 2-chloro-3-oxobutanoate, 2-chloroacetamide, chloroacetonitrile, and iodomethane to afford the corresponding thieno[2,3-b]-pyridine derivatives. Considering the data of IR, ^{1}H NMR, mass spectra and elemental analysis the chemical structures of the newly synthesized heterocyclic compounds elucidated. Cytotoxicity, anti-HSV1, (anti-Herpes Simplex virus type 1) anti-HAV (Hepatitis A virus), and MBB activity were evaluated for the newly synthesized heterocyclic compounds.*

Keywords 2-Cyanoethanethioamide; 2-methyl-thiopyridine-3′-carbonitrile; 2-thioxopyridine; bipyridine-3′-carbonitrile; thieno[2,3-b]pyridine

INTRODUCTION

In continuation to our previous work[1–16] and the reported biological activity of 2-thioxopyridine[17–19] as well as that of thieno[2,3-b]pyridine,[20–21] we were interested in the synthesis of 5′-acetyl-6′-methyl-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′-carbonitrile **(5)** using

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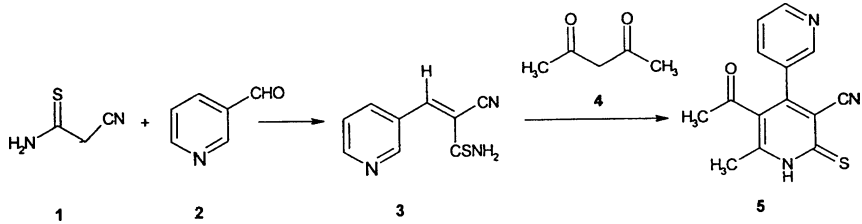
each of 2-cyano-ethanthioamide, pyridine-3-carbaldehyde, and pentan-2,4-dione. The synthetic potentiality of **5** also was investigated through its reactions with several reagents, to synthesize several heterocyclic derivatives, which was required for several chemical transformations as well as for a medicinal chemistry program.

RESULTS AND DISCUSSION

It has been found that 2-cyanoethanethioamide **1** reacted with pyridine-3-carbaldehyde **2** in ethanol containing a catalytic amount of triethyl amine, which gave 2-cyano-3-pyridin-3-ylprop-2-enethioamide **3** that cyclized in methanolic sodium methoxide with pentan-2,4-dione **4** to afford the corresponding 5′-acetyl-6′-methyl-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′-carbonitrile **(5)** according to Scheme 1.

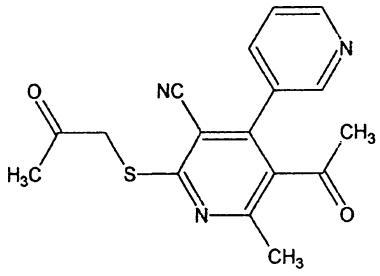


SCHEME 1

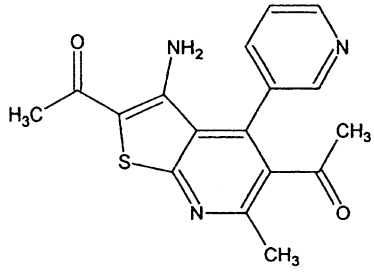
The chemical structure of **5** was elucidated based on its IR, ^{1}H NMR, and its elemental analysis (cf. Experimental section). Moreover, its mass spectrum gave m/z = 269, which corresponds to the molecular weight of the molecular formula $C_{14}H_{11}$ NOS of the assigned structure.

5′-acetyl-6′-methyl-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′-carbonitrile **(5)** reacted with 1-chloroacetone (1:1) in methanolic sodium methoxide solution via dehydrochlorination to afford a reaction product, which formulated as 5′-acetyl-6′-methyl-2′-[(2-oxopropyl)thio]-3,4′-biphenyl-3′-carbonitrile **6** by considering the data of IR, ^{1}H NMR, and elemental analysis (cf. Experimental section). Moreover, its mass spectrum gave m/z = 325, which corresponds to the molecular weight of the molecular formula $C_{17}H_{15}$ N_3O_2S of the assigned structure.

Compound **6** underwent ring closure through its boiling with 10% ethanolic KOH for 3–5 h to give the corresponding 1-(2-acetyl-3-amino-6-methyl-4-pyridin-3-ylthieno[2,3-b]pyridin-5-yl)ethanone **(7)**. The IR spectrum of compound **7** showed no bands corresponding to the CN function, while the newly formed NH_2 was detected in both IR and ^{1}H NMR (cf. Experimental section). The previously-mentioned result



6

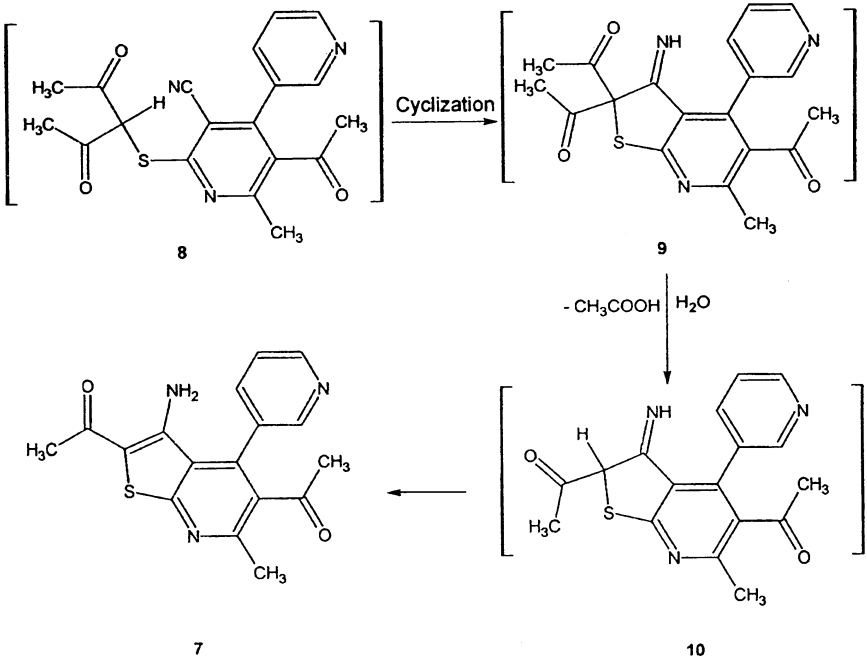


7

SCHEME 2

confirms that both CN and $-CH_2-$ in **6** are involved in the cyclization step to give NH_2 in **7** (Scheme 2).

Compound **5** reacted with 3-chloropentan-2,4-dione via the dehydrochlorination to give **8**, and this was followed by addition to the CN function to give **9**, which then adds one water molecule to yield the corresponding imino derivatives **10** by the elimination of an acetic



8 **9** **10** **7**

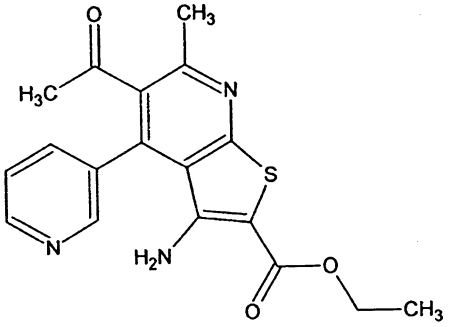
SCHEME 3

acid molecule. The imino derivative **10** will be converted to more stable tautomer **7**.

It is interesting to note that compound **7** obtained by the two pathways is identical in all physical and chemical properties (cf. Experimental section) (Scheme 3).

In a similar reaction, compound **5** reacted with both ethyl chloroacetate and ethyl 2-chloro-3-oxobutanoate to give a reaction product which could be formulated as ethyl 5-acetyl-3-amino-6-methyl-4-pyridin-3-ylthieno[2,3-b]pyridine-2-carboxylate **(11),** whose structure could be elucidated by considering the data of elemental analysis, and IR and ^{1}H NMR. Moreover, the mass spectrum of **11** gave m/z = 355, which corresponds to the molecular weight of the molecular formula $C_{18}H_{17}N_3O_3S$ of the assigned structure.

It is important to report here that compound **5** reacted with ethyl chloroacetate to give a reaction product **11** via the



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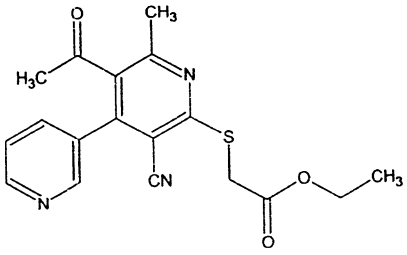

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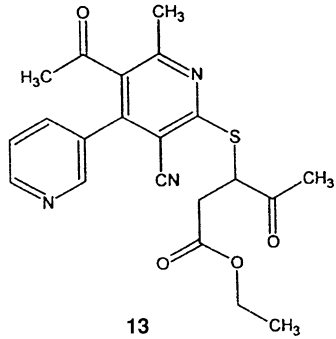
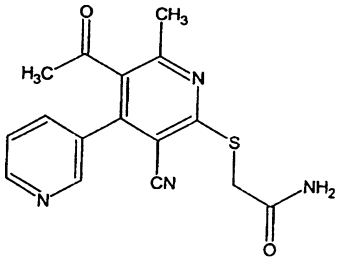


13

SCHEME 4

isolated intermediate ethyl [(5′-acetyl-3′-cyano-6′-methyl-3,4′-bipyridin-2′-yl)thio]acetate **(12)**, while all trials to isolate the intermediate **13** (that **5** reacted with) ethyl 2-chloro-3-oxobutanoate failed (Scheme 4).

Synthon **5** also reacted with 2-chloroacetamide in ethanol containing a catalytic amount of triethyl amine through dehydro-chlorination to give 2-[(5′-acetyl-3′-cyano-6′-methyl-3,4′-bipyridin-2′-yl)thio]acetamide **(14),** which cyclized in 10% ethanolic potassium hydroxide solution to give 5-acetyl-3-amino-6-methyl-4-pyridin-3-ylthieno[2,3- *b*]pyridine-2-carboxamide **(15)**. The IR of **15** showed no bands of a CN function and its ^{1}H NMR did not reveal the signals of CH_2 protons, while the newly formed NH_2 in **15** was detected by both IR and ^{1}H NMR; this confirmed that both CN and CH_2 are involved in cyclization of **14** to give **15** (Scheme 5).



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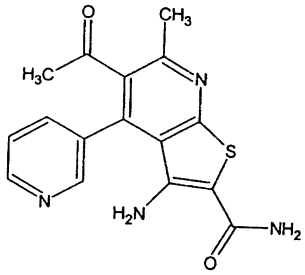
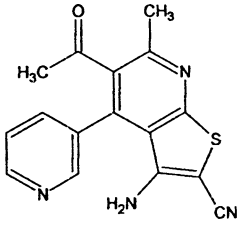


15

SCHEME 5

In a similar investigation, **5** reacted with chloroacetonitrile and iodomethane in methanolic sodium methoxide solution to give 5-acetyl-3-amino-6-methyl-4-pyridin-3-ylthieno[2,3-*b*]pyridine-2-carbonitrile and 5′-acetyl-6′-methyl-2′-(methylthio)-3,4′-bipyridine-3′-carbonitrile **16** and **17**, respectively. The structures (Scheme 6) of **16**



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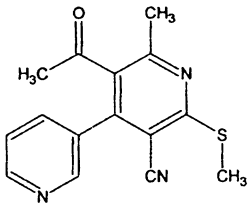

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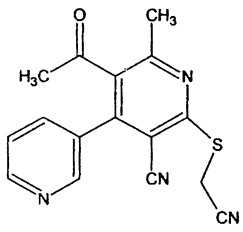

18

SCHEME 6

and **17** were elucidated depending on the data given by elemental analysis, IR, and ^{1}H NMR spectra. Moreover, their mass spectra gave m/z = 308 and 283, respectively, and these correspond to the molecular formulas $C_{16}H_{12}N_4$ OS and $C_{15}H_{13}N_3$OS of the assigned structures.

It is important to note that the reaction of **5** with chloroacetonitrile in methoxide solution proceeded through the non-isolable intermediate **18,** and all trials to isolate it failed. Authentic samples of **7**, **11**, and **15** were prepared through the reaction of **5** with chloroacetone, ethyl chloroacetate, and chloroacetamide, respectively, in 10% ethanolic KOH solution under reflux for 5–7 h. It is important to report here that the isolated compounds **7**, **11**, and **15** are identical in all physical and chemical properties with that given by the cyclization of **6**, **12,** and **14**.

Biological Evaluation

Cytotoxicity assay

As shown in Table (I), compounds **5**, **7**, **9**, **10**, and **12** were much safer for cell culture inoculation than the other compounds, which show CPE at 15 μg. Accordingly, the safe doses were selected and used for antivirus bioassay.

Antivirus bioassay

Screening for Anti-HSV1 Activity. Plaque reduction assay showed that only compound **11** was promising to be an anti-HSV1 (anti-Herpes Simplex Virus Type 1) compound. The percentage of virus reduction

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

	Cytotoxicity grade of the tested materials							
Comp. no.	5 μg	10 μg	15 μg	20 μg	25 μg	30 μg	35 μg	40 μg
5	—	—	—	—	—	—	—	—
6	—	—	—	—	—	+1	+2	+3
7	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—
11	—	—	—	+2	+3	+4	+4	+4
12	—	—	—	—	—	—	+2	+2

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

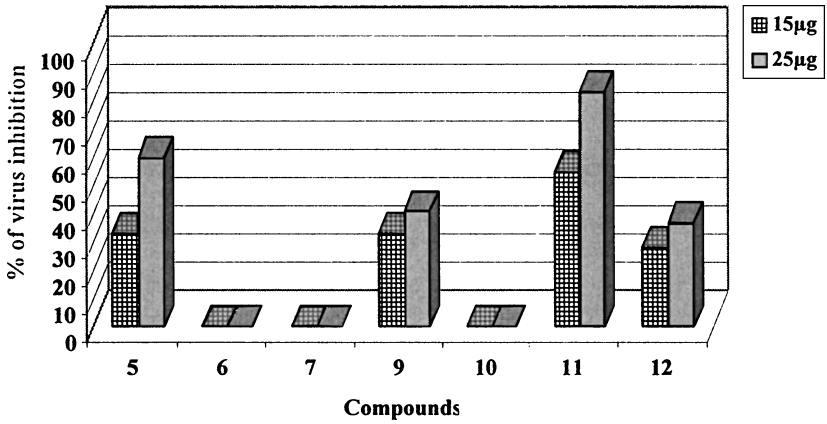

FIGURE 1 Anti-HSV1 bioassay of synthetic compounds in Vero cell line.

was 55% at a dose of 10 μg and increased to 83% with a dose of 15 μg (Figure 1). The compounds **5**, **9**, and **12** exhibited a moderate percentage of virus reduction while all the other compounds had either very weak activity or no activity at all.

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 **7, 10,** and **12** exhibited a moderate percentage of virus reduction while the other newly synthesized heterocyclic compounds either exhibited weak or no activity against Hepatitis a virus (HAV) (Figure 2).

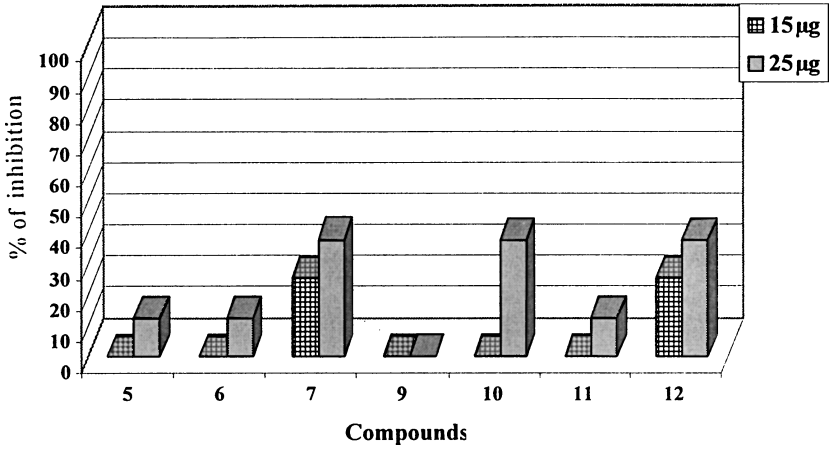

FIGURE 2 Anti-HAV bioassay of synthetic compounds in HepG2 cells.

EXPERIMENTAL

All melting points are uncorrected. IR spectra were recorded as KBr discs on a Shimadzu FTIR-8201PC Spectrophotometer. ^{1}H-NMR spectra were recorded on Varian Mercury 300 MHz and Varian Gemini 200 MHz spectrometers using TMS as an internal standard and $CDCl_3$, DMSO-d_6, and $(CD_3)_2$ CO as solvents. Chemical shifts are expressed 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 microanalyses.

Synthesis of 5′-Acetyl-6′-methyl-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′-carbonitrile (5)

A solution of each of **1** (5 g) and **2** (5.35 g) in absolute ethanol (50 mL) containing a catalytic amount of piperidine (0.4 mL) was stirred at room temperature for 15–20 min. The product formed was filtered off and dried well. The isolated product **3** was obtained as yellow crystals (8.2 g). Mixture of **3** (9.4 g) and **4** (5 g) reacted in absolute ethanol (50 mL) containing a catalytic amount of piperidine under reflux for 5 h. The reaction mixture then was evaporated until it was dry and then cooled. The product formed was collected by filtration, washed with cold ethanol, and then crystallized from ethanol as yellow crystals (9.8 g), m.p. 246–248°C; IR (ν cm^{-1}): 3348 (NH), 2897–2932 (CH-aliphatic); 3051 (pyridine-CH), 2217(CN); 1732 (C=Oester) and 1547 (C=S); ^{1}H-NMR (δppm): 1.5 (s, 3H, CH_3), 2.3 (s, 3H, $\underline{CH_3}$CO), 4.3 (s, 1H, NH) and 7.3–8.6 (m, 4H, pyridine H's); Anal. for $C_{14}H_{11}N_3OS$ (269.32): Calcd./Found (%): C, 62.43/62.4; H, 4.12/4.0; N, 15.60/15.5; S, 11.91/11.3.

Synthesis of 5′-Acetyl-6′-methyl-2′-[(2-oxopropyl)thio]-3,4′-biphenyl-3′-carbonitrile 60

A solution of **5** (6.7 g) and 1-chloroacetone (2.3 g) in methanol containing sodium methoxide (prepared by 0.6 g of sodium in 40 mL methanol) was stirred for 1 h. The product formed was collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give **6** as yellow crystals (6.3 g), m.p. 150°C; IR (ν cm^{-1}): 2887–2922 (CH-aliphatic); 3055 (pyridine-CH), 2219(CN) and 1719 (C=O acetyl); ^{1}H-NMR (δppm): 1.8 (s, 3H, $\underline{CH_3}$ $COCH_2$), 2.1 (s, 3H, CH_3), 2.5 (s, 3H, $\underline{CH_3}$ CO), 4.1(s, 2H, CH_3CO $\underline{CH_2}$) and 7.3–8.6 (m, 4H, pyridine H's); Anal. for C_{17} $H_{15}N_3$ O_2 S (325): Calcd./Found (%): C, 62.75/62.4; H, 4.65/4.2; N, 12.91/12.5; S, 9.85/9.7.

Synthesis of Ethyl [(5′-Acetyl-3′-cyano-6′-methyl-3,4′-bipyridin-2′-yl)thio]acetate 12

A solution of **5** (6.7 g) and ethyl chloroacetate (3.3 g) in methanol containing sodium methoxide (prepared by 0.6 g of sodium in 40 mL methanol) was stirred for 1 h. The product formed was collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give **12** as pale orange crystals (6.6 g), m.p. 204°C; IR (ν cm^{-1}): 2889–2931 (CH-aliphatic); 3062 (pyridine-CH), 2222(CN) 1735 (C=O ester) and 1713 (C=O acetyl); ^{1}H-NMR (δppm): 1.2 (t, 3H, $\underline{CH_3}$ CH_2-), 2.1 (s, 3H, CH_3), 2.6 (s, 3H, $\underline{CH_3}$ CO), 3.7 (s, 2H, -$\underline{CH_2}$ $COOC_2$ H_5), 4.5 (q, 2H, CH_3 $\underline{CH_2}$-) and 7.1–8.7 (m, 4H, pyridine H's); Anal. for C_{18} H_{17} N_3 O_3 S (355): Calcd./Found (%): C, 60.83/60.7; H, 4.82/4.7; N, 11.82/11.9; S, 9.02/9.1.

Synthesis of 2-[(5′-Acetyl-3′-cyano-6′-methyl-3,4′-bipyridin-2′-yl)thio]-acetamide 14

A solution of **5** (6.7 g) and chloroacetamide (2.3 g) in methanol containing sodium methoxide (prepared by 0.6 g of sodium in 40 mL methanol) was stirred for 1 h. The product formed was collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give **14** as pale brown crystals (6.4 g), m.p. 165°C; IR (ν cm^{-1}): 3432, 3378, 3344 (NH_2), 2882–2921 (CH-aliphatic); 3052 (pyridine-CH), 2220(CN) 1715 (C=O acetyl) and 1693 (C=O amide); ^{1}H-NMR (δ ppm): 2.0 (s, 3H, CH_3), 2.5 (s, 3H, $\underline{CH_3}$CO), 3.8 (s, 2H, —$\underline{CH_2}$ $CONH_2$), 6.1 (s, br., 2H, NH_2) and 6.9–8.6 (m, 4H, pyridine H's); Anal. for $C_{16}H_{14}N_4O_2S$ (326): Calcd./Found (%): C, 58.55/58.4; H, 4.32/4.2; N, 17.17/17.2; S, 9.82/9.8.

Synthesis of 5′-acetyl-6′-methyl-2′-(methylthio)-3,4′-bipyridine-3′-carbonitrile 17

A solution of **5** (6.7 g) and iodomethane (4.7 g) in methanol containing sodium methoxide (prepared by 0.6 g of sodium in 40 mL methanol) was stirred for 1 h. The product formed was collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give **17** as pale yellow crystals (4.9 g), m.p. 188°C; IR (ν cm^{-1}): 2885–2933 (CH-aliphatic); 3059 (pyridine-CH), 2219(CN) and 1718 (C=O acetyl); ^{1}H-NMR (δppm): 1.9 (s, 6H, two CH_3), 2.5 (s, 3H, $\underline{CH_3}$CO) and 7.1–8.8 (m, 4H, pyridine H's); Anal. for $C_{15}H_{13}N_3OS$ (283): Calcd./Found (%): C, 63.58/63.4; H, 4.62/4.7; N, 14.83/14.9; S, 11.32/11.4.

Synthesis of 1-(2-Acetyl-3-amino-6-methyl-4-pyridin-3-ylthieno[2,3-b]pyridin-5-yl)ethanone 7

First Method

A solution of **5** (6.7 g) and 1-chloroacetone (2.3 g) in ethanol (30 mL) and KOH 10% solution (10 mL) was heated under reflux for 3 h. The reaction mixture was cooled, poured onto ice-cold water, and acidified with hydrochloric acid; the product formed was collected by filtration, washed with water followed by cold ethanol, and recrystallized from ethanol, to give **7**.

Second Method: (Cyclization of 6)

A solution of **6** (3.6 g) in ethanol (30 mL) and KOH 10% solution (10 mL) was heated under reflux for 3 h. The reaction mixture was cooled, poured into ice-cold water, and acidified with hydrochloric acid; the product formed was collected by filtration, washed with water followed by cold ethanol, and recrystallized from ethanol to give **7** as yellowish white crystals (2.1 g), m.p. 190°C; IR (ν cm^{-1}): 3432, 3389, 3332 (NH_2), 2894–2938 (CH-aliphatic); 3073 (pyridine-CH), and 1715 (C=O acetyl); ^{1}H-NMR (δppm): 2.6 (s, 9H, three CH_3), 4.3 (s, br., 2H, NH_2) and 7.2–8.7 (m, 4H, pyridine H's); Anal. for C_{17} $H_{15}N_3O_2S$ (325): Calcd./Found (%): C, 62.75/62.8; H, 4.65/4.7; N, 12.91/12.7; S, 9.83/9.9.

Synthesis of Ethyl 5-Acetyl-3-amino-6-methyl-4-pyridin-3-ylthieno-[2,3-b]pyridine-2-carboxylate 11

First Method

A solution of **5** (6.7 g) and ethyl chloroacetate (3.1 g) or ethyl 2-chloro-3-oxobutanoate (4.1 g) in ethanol (30 mL) and KOH of a 10% solution (10 mL) was heated under reflux for 3 h. The reaction mixture was cooled, poured onto ice-cold water, and acidified with hydrochloric acid; the product formed was collected by filtration, washed with water followed by cold ethanol, and recrystallized from ethanol to give **11**.

Second Method: (Cyclization of 12)

A solution of **12** (3.9 g) in ethanol (30 mL) and KOH 10% solution (10 mL) was heated under reflux for 3 h. The reaction mixture was cooled, poured into ice-cold water, and acidified with hydrochloric acid, the product formed was collected by filtration, washed with water followed by cold ethanol, and recrystallized from ethanol to give **11** as pale brown crystals (2.5 g), m.p. 187°C; IR (ν cm^{-1}): 3443, 3388, 3342 (NH_2), 2887–2925 (CH-aliphatic); 3062 (pyridine-CH), 1732 (C=O ester) and 1712 (C=O acetyl); ^{1}H-NMR (δppm): 1.2 (t, 3H, $\underline{CH_3}$ $CH_{\underline{2}}$–), 2.5 (s, 6H,

two CH_3), 3.9 (s, br., 2H, NH_2), 4.5 (q, 2H, CH_3 $\underline{CH_2}$—) and 7.0–8.6 (m, 4H, pyridine H's); Anal. for $C_{18}H_{17}N_3O_3$ S (355): Calcd./Found (%): C, 60.83/60.8; H, 4.82/4.7; N, 11.82/11.7; S, 9.02/9.1.

Synthesis of 5-Acetyl-3-amino-6-methyl-4-pyridin-3-ylthieno[2,3-b-pyridine-2-carboxamide 15

First Method

A solution of **5** (6.7 g) and chloroacetamide (2.3 g) in ethanol (30 mL) and KOH 10% solution (10 mL) was heated under reflux for 3 h. The reaction mixture was cooled, poured onto ice-cold water, and acidified with hydrochloric acid; the product formed was collected by filtration, washed with water followed by cold ethanol, and recrystallized from ethanol, to give **15**.

Second Method: (Cyclization of 14)

A solution of **14** (3.6 g) in ethanol (30 mL) and KOH 10% solution (10 mL) was heated under reflux for 3 h. The reaction mixture was cooled, poured into ice-cold water, and acidified with hydrochloric acid; the product formed was collected by filtration, washed with water followed by cold ethanol, and recrystallized from ethanol to give **15** as white crystals (2.6 g), m.p. 248°C; IR (ν cm^{-1}): 3454, 3394, 3364 (NH_2), 2889–2932 (CH-aliphatic); 3056 (pyridine-CH), 1713 (C═O acetyl) and 1697 (C═O amide); ^{1}H-NMR (δppm): 2.0 (t, 3H, CH_3), 2.5 (s, 3H, CH_3), 4.0 (s, br., 2H, NH_2), 6.2 (s, br., 2H, CO $\underline{NH_2}$) and 7.3–8.9 (m, 4H, pyridine H's); Anal. for $C_{18}H_{17}N_3O_3S$ (326): Calcd./Found (%): C, 58.88/58.7; H, 4.32/4.5; N, 17.17/17.2; S, 9.82/9.9.

Synthesis of 5-Acetyl-3-amino-6-methyl-4-pyridin-3-ylthieno[2,3-b-pyridine-2-carbonitrile 16

A solution of **5** (2.9 g) and chloroacetonitrile (0.8 g) in methanolic sodium methoxide (0.3 g of sodium in 30 mL methanol) was stirred for 1–2 h or was heated under reflux for 20–30 min. The product was formed, filtrated off, washed with cold ethanol, and recrystallized from ethanol to give **16** as dark brown crystals (2.7 g), m.p. 110°C; IR (ν cm^{-1}): 3433, 3389, 3367 (NH_2), 2884–2927 (CH-aliphatic); 3054 (pyridine-CH), 2220 (CN) and 1717 (C═O acetyl); ^{1}H-NMR (δppm): 2.0 (t, 3H, CH_3), 2.5 (s, 3H, CH_3), 4.3 (s, br., 2H, NH_2) and 7.1–8.7 (m, 4H, pyridine H's); Anal. for C_{16} $H_{12}N_4OS$ (308): Calcd./Found (%): C, 62.32/62.4; H, 3.92/4.1; N, 18.17/18.0; S, 10.40/10.5.

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 were: African green monkey kidney-derived cells (VERO), cell line obtained from American type culture collection (ATCC), and human hepatoma cell line (Hep G2).

Viruses

Two models of DNA & RNA viruses were used for bioassay. These viruses were HSV-1 and HAV, MBB strain (HAN-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 0.22 μm of 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 10% final concentration for a growth medium and at 2% for a maintenance medium.

Antibiotic–Antimycotic Mixture (GIBCO-BRL)

100 × antibiotic–antimycotic mixtures consisted of 10,000 U penicillin G sodium, 10,000 μg streptomycin sulfate, and 25 μg 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), and deionized H_2O (up to 1 L). Ingredients were mixed gently in this order 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 in the washing of cell monolayer sheets and in preparation of 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 was dissolved in 500 mL of 0.15 M PBS pH 7.5 to prepare 2 mM solution (0.04 gm) and mixed with equal volume of trypsin solution. The pH value of the trypsin–versene mixture was adjusted at 8.4 by a 7.5% sodium bicarbonate solution and sterilized by filtration through a 0.22-μm pore-size nitrocellulose membrane. 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% Dimethyl sulfoxide (DMSO) in water. The final concentration was 100 μg/μl (stock solution). The dissolved stock solutions were sterilized by the addition of an antibiotic–antimycotic mixture: 10,000 U penicillin G sodium or Gentamicin 50 μg/mL; 10,000 μg streptomycin sulfate; and 250 μg amphotericin B. Sterility test were carried out in nutrient agar.

II: Cell Culture

VERO were used. The cells were Propagated in Hanks' MEM, and supplemented with 10% FBS, and 1% antibiotic-antimycotic mixture. The pH was adjusted at 7.2–7.4 by a 7.5% sodium bicarbonate solution. The mixture was sterilized by filtration through a 0.22 μm pore-size nitrocellulose membrane.

III: Viruses

HSV-1, obtained from Environmental Virology lab Department of Water Pollution Res., National Research Centre, Dokki, Egypt.

IV: Antiviral Assay

Cytotoxicity Assay. Cytotoxicity was assayed for both 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 it with trypan blue dye.

Plaque Reduction Assay. A 6-well plate was cultivated with a VERO cell culture (10^5 cell/mL) and incubated for 2 days at 37°C. HSV-1 was diluted to give a 10^4 PFU/mL final concentration and mixed with the plant extract at the previous concentration and incubated overnight at 4°C. Growth medium was removed from the multiwell plate and a virus–compound mixture was inoculated (100 μl/well). After 1 h contact

time, the inoculum was aspirated and 3 mL of MEM with 1% agar rose 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. The 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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