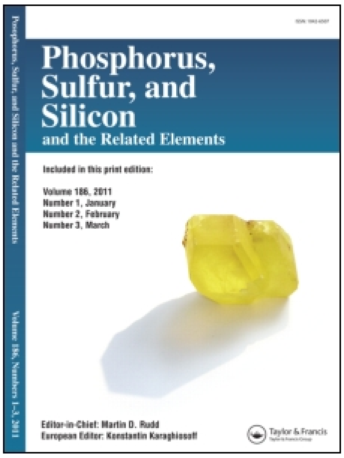

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Synthesis, Reactions, and Antiviral Activity of 6'-Amino-2'-thioxo-1',2'-dihydro-3,4'-bipyridine-3',5'-dicarbonitrile

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Synthesis, Reactions, and Antiviral Activity of 6′-Amino-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′,5′-dicarbonitrile

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*Nicotinaldehyde **1** reacted with 2-cyanoethanethioamide **2** to give 2-cyano-3-pyridin-3-ylprop-2-enethioamide **3**, which reacted with a second mole of **2** to give the corresponding 6′-amino-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′,5′-dicarbonitrile **6**. The synthetic potentiality of compound **6** was investigated via its reaction with active halogen-containing reagents, e.g., chloroacetone, 2-chloro-3-oxobutanoate, 2-chloroacetamide, chloroaceto-nitrile, and ethyl chloroacetate, to afford the corresponding thieno[2,3-b]-pyridine derivatives. Structure elucidation of all newly synthesized heterocyclic compounds was based on the data of elemental analyses, and IR, ^{1}H NMR, as well as mass spectra. Cytotoxicity, anti–HSV1, anti–HAV, and MBB activities were evaluated for all newly synthesized heterocyclic compounds.*

Keywords 2-Cyano-3-pyridin-3-yl-prop-2-enethioamide; 2-cyanoethanethioamide; bipyridine-3′, 5′-dicarbonitrile; nicotinaldehyde; thieno[2,3-b]pyridine

INTRODUCTION

The reported biological activity of 2-thioxopyridine,[1–3] and thienopyridine,[4,5] and the conjunction of our previous work[5–23] stimulate our interest to synthesize several derivatives of these ring systems required for chemical transformations and a medicinal chemistry program. We also are interested in investigating the chemical reactivity and synthetic potentiality of both —CH=C— in **3** and —CH$_2$— in **2**.

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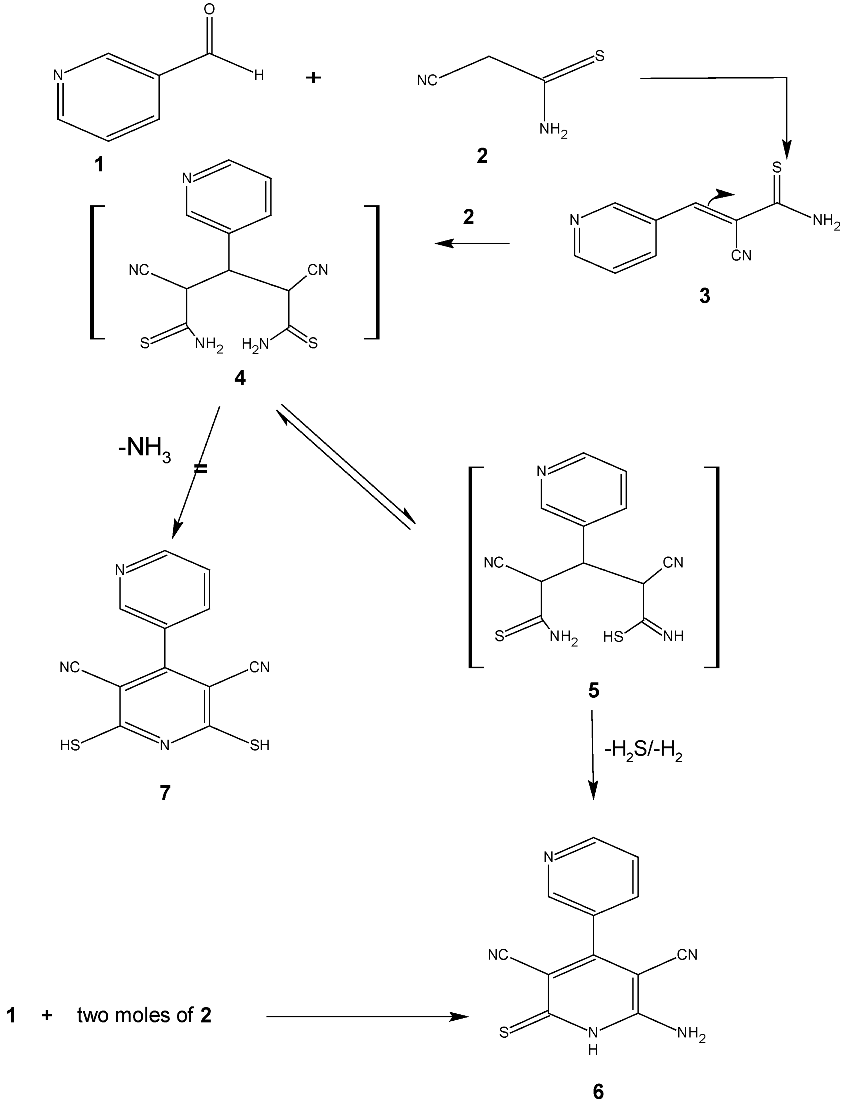
RESULTS AND DISCUSSION

It has been found that nicotinaldehyde **1** reacted with 2-cyanoethanethioamide **2** to afford 2-cyano-3-pyridin-3-ylprop-2-enethioamide **3** that reacted in turn with a second mole of **2** to give **6** via the non-isolable structures **4** and **5**. The IR (cm^{-1}) of this reaction product showed the bands of NH_2, NH, and CN functions, and ^1H NMR (δ ppm) revealed the signals of NH_2, NH, and pyridine protons. Moreover, its mass spectrum gave m/z = 253, which corresponded to the molecular weight of the molecular formula $C_{12}H_7N_5S$ of the assigned structure (cf. Experimental section and Scheme 1). By considering the previously mentioned data in addition to the data of elemental analyses, we rolled out structure **7** and conclude that this reaction product underwent auto-oxidation under the experimental conditions to afford **6**, which was formulated as 6′-amino-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′,5′-dicarbo-nitrile **6** (cf. Scheme 1) and Experimental section). Authentically **6** was obtained also by a reaction of both **1** and **2** in the ratio 1:2 as a ternary mixture.

The synthetic potential of **6** was investigated through its reaction with haloketones, haloesters, halonitriles, and haloalkanes. Thus, it has been found that **6** reacted with chloroacetone (**8a**) in methanolic sodium methoxide to afford a reaction product formed via dehydrochlorination. The IR of this reaction product showed bands of NH_2, CN, and acetyl CO functions; its ^1H NMR spectrum revealed signals of $\underline{CH_3}CO$, NH_2, and pyridine protons; moreover, its mass spectrum gave a peak at m/z = 309, which corresponded to the molecular weight of the molecular formula $C_{15}H_{11}N_5SO$ of the assigned structure (cf. Experimental section). Considering the previously mentioned data in addition to that of elemental analyses, this reaction product was obtained through the non-isolable products **9a** and **10a** and was formulated as 2-acetyl-3,6-diamino-4-pyridin-3-ylthieno[2,3-*b*]pyrid-ine-5-carbonitrile (**11**) (cf. Scheme 2).

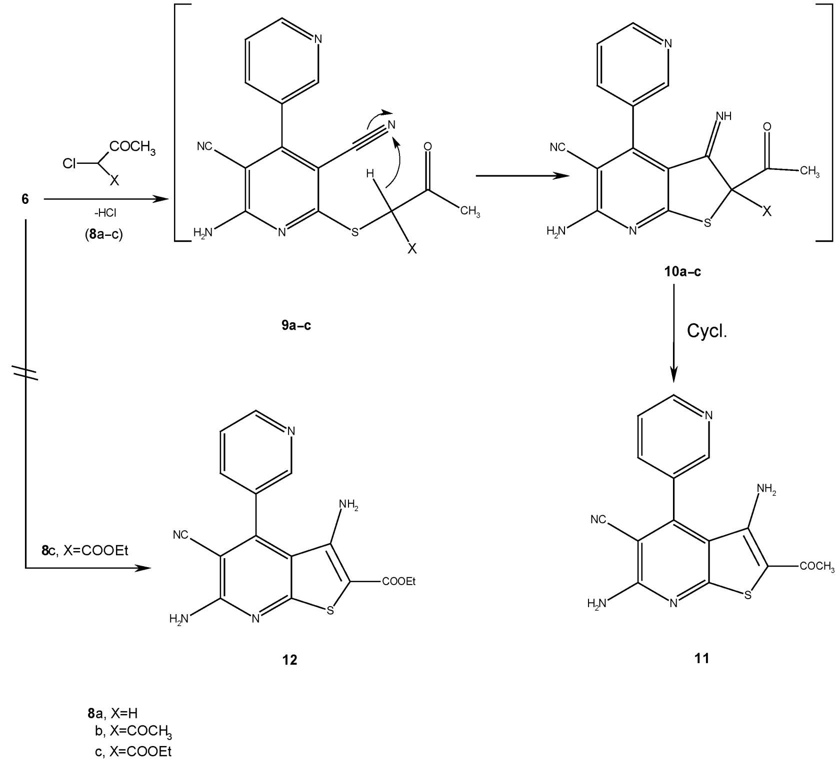
The reaction product **11** was also obtained through different pathways. Thus, it has been found that **6** reacted with each of 3-chloropentan-2,4-dione and ethyl 2-chloro-3-oxobutanoate **8b**, **c** under the same previously mentioned experimental conditions through the non-isolable products **9b**, **c** and **10b**, **c**, respectively (cf. Scheme 2).

Structure **12** was not formed via the reaction of **6** with **8c** depending on the data of ^1H NMR, which revealed signals of $CO\underline{CH_3}$ protons and did not reveal signals of $COO\underline{CH_2CH_3}$ protons. This also confirmed using the data of mass spectra, which did not give m/z = 339, which corresponded to the molecular weight of the molecular formula $C_{16}H_{13}N_5O_2S$ of the assigned structure **12**, while it gave a peak at



SCHEME 1

m/z = 309, which corresponded to the molecular weight of the molecular formula $C_{15}H_{11}N_5OS$ of the assigned structure **11**. It is important to report here a that compound **11** obtained via **8b** formed through addition of H_2O molecule followed by the removal of the acetic acid molecule. On the other hand, **11** formed via **8c** through hydrolysis with ethyl

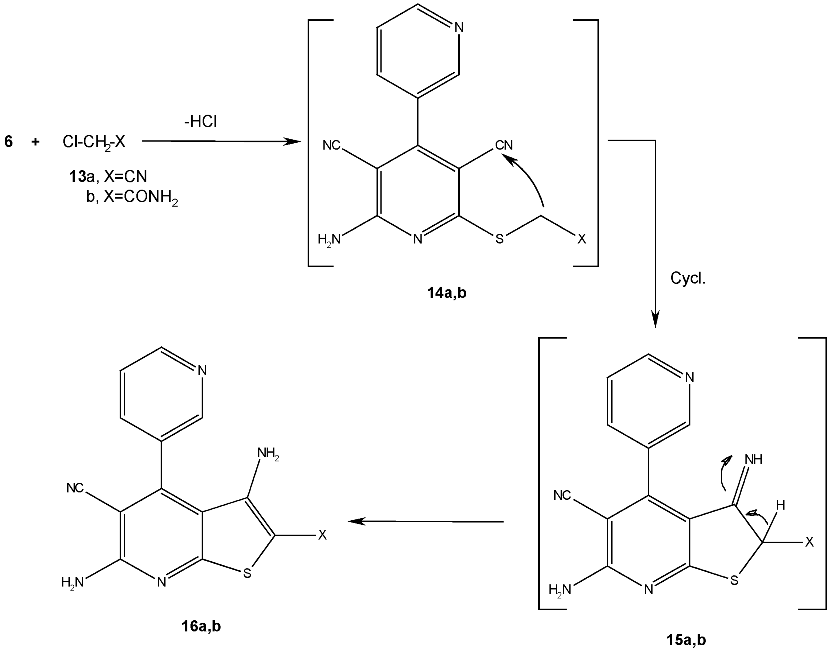


SCHEME 2

alcohol molecule removal, and this was followed by decarboxylation (cf. Experimental section).

In further investigation, compound **6** reacted with each of chloroacetonitrile and 2-chloroacetamide **13a**, **b** in a ratio of 1:1 in methanolic sodium methoxide through dehydrochlorination to afford reaction products with the molecular formulas $C_{14}H_8N_6S$ and $C_{14}H_{10}N_6SO$, respectively. The IR (cm^{-1}) of these reaction products showed bands of NH_2 and CN functions; their ^{1}H NMR spectra revealed signals of NH_2 and pyridine protons. Moreover, their mass spectra gave m/z = 292 and 310, which corresponded to the molecular weights of the molecular formulas $C_{14}H_8N_6S$ and $C_{14}H_{10}N_6SO$ of the assigned structures **16a**,**b**. The formation of **16a**,**b** proceeded via the non-isolable products **14a**,**b** and **15a**, **b**, respectively (cf. Scheme 3 and Experimental section).

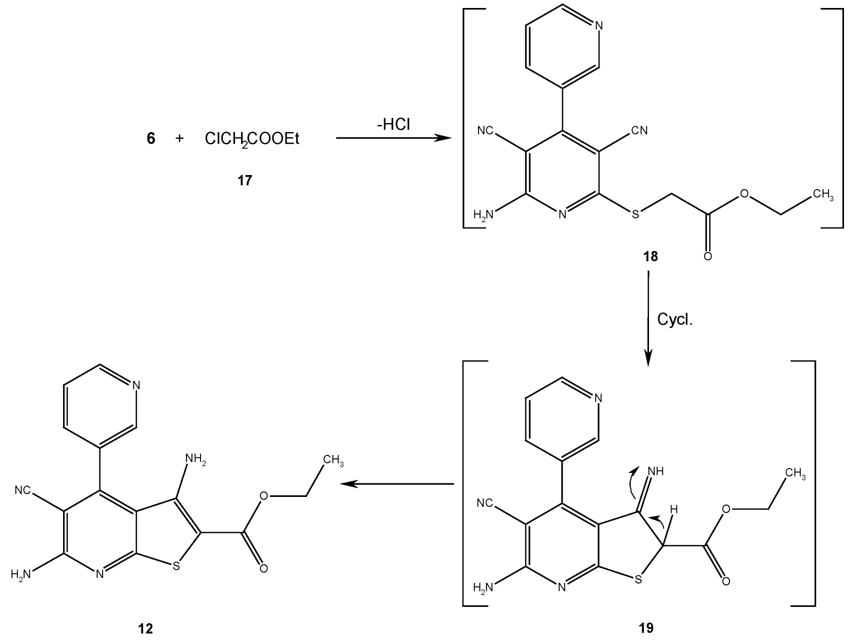
The chemical reactivity and synthetic potentiality of **6** were further investigated through its reaction with ethyl chloroacetate (**17**) to afford a reaction product formed by dehydrochlorination. The IR of this reaction product showed the bands of NH_2, CN, and CO ester functions; its



SCHEME 3

^{1}H NMR revealed the signals of $\underline{CH}_3CH_2$, $CH_3\underline{CH}_2$, NH_2, and pyridine protons. Moreover, its mass spectrum gave the parent peak at m/z = 339 (27.2%), which corresponded to the molecular weight of the molecular formula $C_{16}H_{13}N_5O_2S$ in addition to the base peak at m/z = 266 (100%), which corresponded to M-73 (elimination of the COOEt fragment from the parent peak). Considering the previously mentioned data and the data of elemental analyses, we can conclude that the reaction product and it represented as **12** formed through the non-isolable products **18** and **19**, and it could be formulated as ethyl 3,6-diamino-5-cyano-4-pyridin-3-ylthieno[2,3-*b*]pyridine-2-carboxylate (cf. Scheme 4).

The synthetic potentiality of **6** could be established through its reaction with iodomethane followed by its reaction with hydrazine hydrate. Thus, it has been found that **6** reacted with iodomethane in methanolic sodium methoxide to afford a reaction product formed through dehydroiodonation. The IR spectrum of this reaction product showed bands of NH_2, CN, and aliphatic CH (no bands for NH function); its ^{1}H NMR spectrum revealed the signals of CH_3, NH_2, and pyridine protons. Furthermore, its mass spectrum gave m/z = 267, which corresponded to the molecular weight of the molecular formula $C_{13}H_9N_5S$ of the assigned



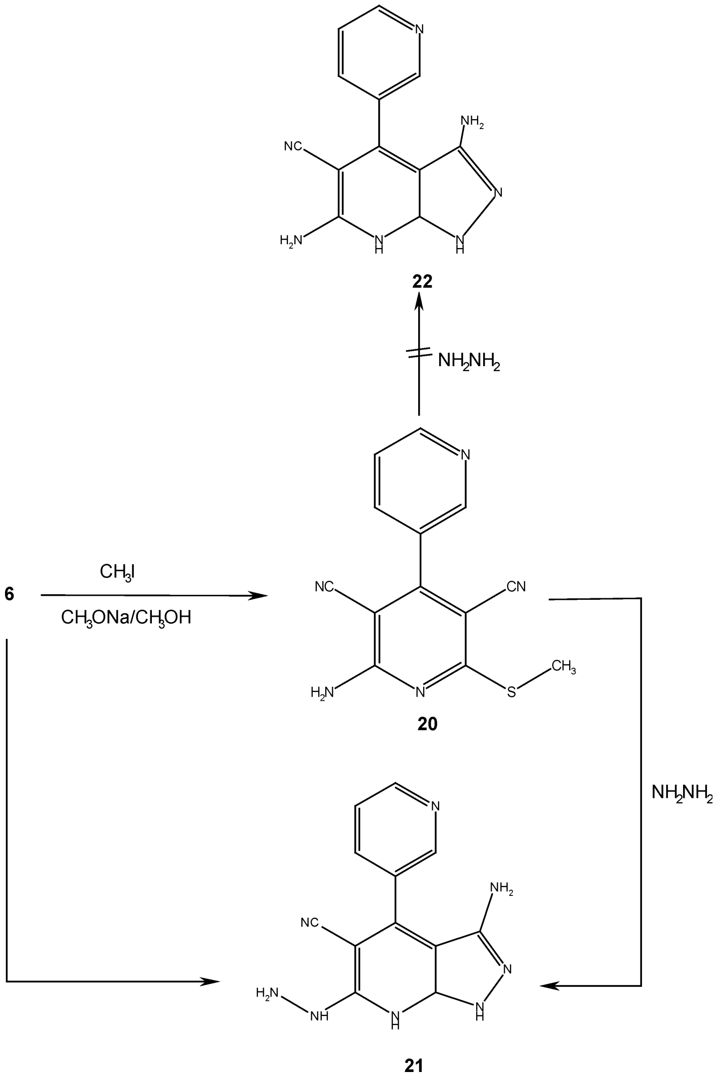
SCHEME 4

structure **20**. Compound **20** reacted with hydrazine hydrate to give a sulfur-free reaction product whose structure was established by considering the data of IR, ^{1}H NMR, mass spectra, and elemental analyses (cf. Experimental section). The element test of this reaction product gave no S, and this proves the removal of the SCH_3 group during the reaction with hydrazine hydrate; this also confirms, through the signal absence of SCH_3, protons from the ^{1}H NMR spectrum. Moreover, the mass spectrum of this reaction product gave m/z = 267, and this confirms, that NH_2 at the 6-position reacted with hydrazine hydrate to give $–NHNH_2$ instead of NH_2 so that the reaction product could be formulated as 3-amino-6-hydrazino-4-pyridin-3-yl-7,7a-dihydro-1*H*-pyrazolo[3,4-*b*]pyridine-5-carbonitrile **21**, not **22**. Compound **21** could be prepared authentically through the reaction of **6** with hydrazine hydrate (cf. Scheme 5).

Biological Evaluation

Cytotoxicity Assay

As shown in Table I, compounds **12**, **16a**, **b**, and **21** were much safer for cell culture inoculation than the other compounds. Compound **11** showed high cytotoxicity at doses 15–45 μg, while all other compounds



SCHEME 5

showed CPE at 20 μg. Accordingly, the safe doses were selected and used for anti-virus bioassay.

Antivirus Bioassay

Screening for Anti-HSV1 Activity

Plaque reduction assay showed that compounds **6**, **11**, **16a**, **20**, and **21** were promising to be anti-HSV1 compounds. The percentage of virus

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

Compound No.	Cytotoxicity Grade of the Tested Materials (μg) 5	10	15	20	25	30	35	40
6	—	—	—	—	—	+1	+2	+3
11	+2	+3	+4	+4	+4	+4	+4	+4
12	—	—	—	—	—	—	—	—
16a	—	—	—	—	—	—	—	—
16b	—	—	—	—	—	—	—	—
20	—	—	—	—	+1	+1	−1	+1
21	—	—	—	—	—	—	—	—

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

reduction was 35% and increased to 57% at dose 25 μg, and the same compounds showed a moderate anti-HSV1 activity at dose 15 μg. Compound **16b** showed no activity at dose 25 μg and showed 35% of virus reduction at dose 15 μg, while compound **12** exhibited no activity at all (Figure 1).

Screening for Anti-HAV and MBB Strain Activity

All compounds were tested for anti–Hepatitis A virus activity in HepG2 cells by plaque reduction assay. The results shown that compounds **6** and **21** exhibited a moderate percentage of virus reduction at

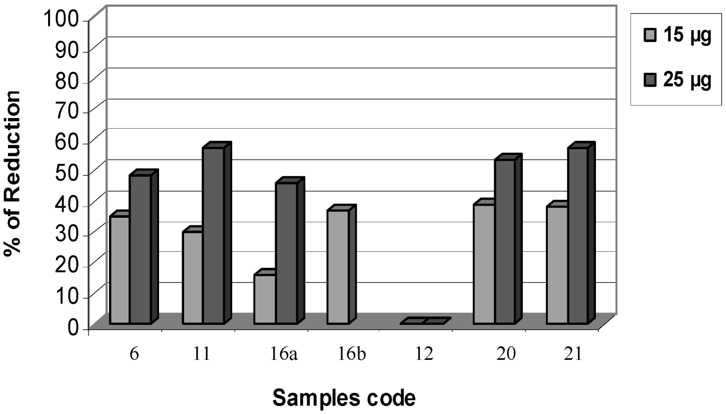


FIGURE 1 Anti-HSV1 bioassay of synthetic compounds in Vero cell line.

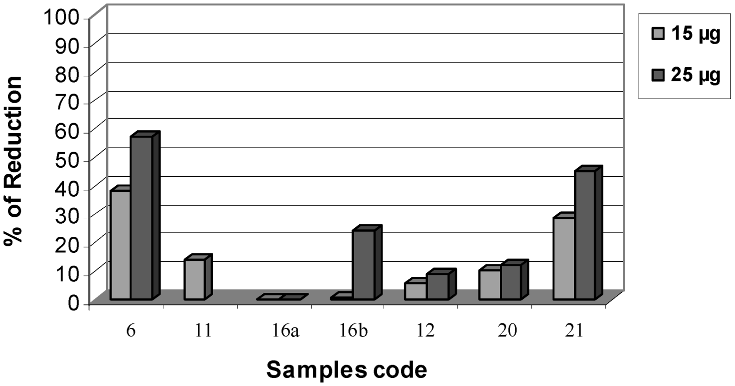


FIGURE 2 Anti-HAV bioassay of synthetic compounds in HepG2 cells.

dose 15 μg and high activity at dose 25 μg, while the other newly synthesized heterocyclic compounds either exhibited weak or no activity against HAV (Figures 1 and 2).

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 DMSO as solvents. Chemical shifts are expressed as δ ppm units.

Mass spectra were recorded on Shimadzu GCMS-QP1000EX using inlet type at 70 eV. The Microanalytical Center of Cairo University (Giza, Egypt) performed microanalyses.

Synthesis of 6′-Amino-2′-thioxo-1′,2′-dihydro-3,4′-bipyridine-3′,5′-dicarbonitrile (6)

A solution of each of **1** (3 mmole, 3.21 g) and **2** (3 mmole, 3 g) in absolute ethanol (50 mL) containing a catalytic amount of piperidine (0.4 mL) was stirred at r.t. for 15–20 min. The product that formed was filtered off and dried well. The isolated product **3** was obtained as yellow crystals. A mixture of **3** (3 mmole, 5.67 g) and **2** (3 mmole, 3 g) reacted in absolute ethanol (50 mL) containing a catalytic amount of piperidine under reflux for 5 h. The reaction mixture was then evaporated until dryness and then cooled. The product formed was collected by filtration, washed with cold ethanol, and then crystallized from ethanol as yellow crystals, m.p. 246–248°C; **IR** (**ν cm^{-1}**): 3455, 3348, 3155 (NH_2 and NH), 3071 (pyridine-CH), 2213(CN at C-3), 2205(CN at C-5) and 1550 (C=S); **^{1}H NMR** (**δ ppm**): 4.2 (s, 2H, NH_2), 5.3 (s, br., 1H, <u>NH</u>) and 7.3–8.7 (m, 4H, pyridineH's); **mass spectra** (**m/z, intensity** %): parent peak (m/z = 253, 54%), base peak (m/z = 84, 100%, due to the fragment NC—CH=C—SH), peak at m/z = 209, 9.8% due to the loss of CS from the parent), the latter peak lost NH to afford peak at m/z = 194 (9.0%) that lost CH_2N fragment to give peak at m/z = 166 (19.3%) that lost a C_2NH fragment to give peak at m/z = 131 (14.3%); anal. for $C_{12}H_7N_5S$ (253.28): calcd./found (%): C, 56.90/57.0; H, 2.79/2.8; N, 27.65/27.5; S,12.66/12.7.

Synthesis of 11

A solution of 6 (0.5 mmole, 1.27 g) and **8a** (0.5 mmole, 0.46 g), **8b** (0.5 mmole, 0.67 g), or **8c** (0.5 mmole, 0.82 g) in methanol containing sodium methoxide (prepared by 0.6 g of sodium in 40 mL methanol) was stirred for 5 h. The product that formed was collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give **2-acetyl-3,6-diamino-4-pyridin-3-ylthieno[2,3-b]pyridine-5-carbonitrile** (**11**) as yellow crystals, m.p. >300°C; **IR** (**ν cm^{-1}**): 3456, 3344, 3188 (NH_2); 2873–2950 (CH-aliphatic); 3048 (pyridine-CH), 2215(CN) and 1724 (C=O acetyl); **^{1}H-NMR** (**δ ppm**): 2.3 (s, 3H, CH_3CO), 5.1(s, br., 2H, NH_2) and 7.1–8.5 (m, 4H, pyridine H's); **mass spectra** (**m/z**, **intensity** %) parent peak and also the base peak (intensity 100%) at m/z = 309, peak at m/z = 266 (36.9%), which corresponded to M-43 (elimination of CH_3CO from the parent peak), the latter peak lost a CS fragment to give peak at m/z = 222 (5.9%) that lost a fragment of NH_2-CN to afford peak at m/z = 180 (17.3%) that also underwent fragmentation to give peaks at m/z = 142 (8.4%) and 102 (7.3%). Anal. for $C_{15}H_{11}N_5OS$ (309): calcd./found (%): C, 58.24/58.2; H, 3.58/3.6; N, 22.64/22.5; S, 10.37/10.4.

Synthesis of 12

A solution of **6** (0.5 mmole, 1.27 g) and ethyl chloroacetate **17** (0.5 mmole, 0.61 g) in methanol containing sodium methoxide (prepared by 0.6 g of sodium in 40 mL methanol) was stirred for 5 h. The product that formed was collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give **ethyl 3,6-diamino-5-cyano-4-pyridin-3-ylthieno[2,3-b]pyridine-2-carboxylate** (**12**) as yellow crystals, m.p. 306–308°C; IR (ν cm^{-1}): 3451, 3340, 3190 (NH_2); 3050 (pyridine-CH); 2218 (CN) and 1735 (CO ester); **^{1}H NMR (**δ **ppm)**: 1.5

(t, 3H, $COOCH_2\underline{CH_3}$); 4.3 (q, 2H, $COO\underline{CH_2}CH_3$); 5.2(s, br., 4H, two NH_2) and 7.3–8.6 (m, 4H, pyridine H's); **mass spectra (m/z, intensity %)** parent peak at m/z = 338 (47.6%), base peak at m/z = 266 (100%) in addition to several peaks of low intensity; anal. for $C_{16}H_{13}N_5SO_2$ (339.3): calcd./found (%): C, 56.63/56.7; H, 3.86/3.9; N, 20.64/21.0; S, 9.45/9.4.

Synthesis of 16a,b

A solution of **6** (0.5 mmole, 1.27 g) and chloroacetonitrile **13a** (0.5 mmole, 0.38 g) and 2-chloroacetamide **13b** (0.5 mmole, 0.47 g) in methanol containing sodium methoxide (prepared by 0.6 g of sodium in 40 mL methanol) was stirred for 5 h. The products that formed were collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give **16a**,**b**, respectively.

3,6-diamino-4-pyridin-3-ylthieno[2,3-b]pyridine-2,5-dicarbonitrile (**16a**) as brown crystals, m.p. >300°C; **IR** (ν **cm**$^{-1}$): 3474, 3354, 3194 (NH_2); 3051 (pyridine-CH) and 2220(CN); ^{1}**H-NMR (**δ **ppm)**: 5.2(s, br., 4H, two NH_2) and 7.3–8.6 (m, 4H, pyridine H's); **mass spectra (m/z, intensity %)** parent peak at m/z = 292 (70.1%) that lost hydrogen to afford the base peak at m/z = 291 (100%) that lost CN to give peak at m/z = 266 (3.3%) and peak at m/z = 194 (3.8%) due to the loss of NH_2—C=C—S—CN. Anal. for $C_{14}H_8N_6S$ (292.3): calcd./found (%): C, 57.52/57.6; H, 2.76/2.8; N,28.75/28.5; S;10.97/10.1.

3,6-diamino-5-cyano-4-pyridin-3-ylthieno[2,3-b]pyridine-2-carboxa-mide (**16b**) as yellow crystals, m.p. >300°C; **IR** (ν **cm**$^{-1}$): 3466, 3342, 3189 (NH_2); 3055 (pyridine-CH); 2222 (CN) and 1675 (CO amidic); ^{1}**H NMR** (δ **ppm**): 4.8 (s, br., 4H, two NH_2); 5.7 (s, br., 2H, $CO\underline{NH_2}$) and 7.2–8.4 (m, 4H, pyridine H's); **mass spectra (m/z, intensity %)** parent peak at m/z = 310 (56.4%) that lost H_2O to give the base peak at m/z = 292 (100%), the latter peak lost HCN, and —CN, to give peaks at m/z = 265 (13.6%) and at m/z = 266 (8.5%); anal. for $C_{14}H_{10}N_6SO$ (310.3): calcd./found (%): C, 54.18/54.2; H, 3.25/3.3; N, 27.08/27.1; S, 10.33/10.4.

Synthesis of 2′-Amino-6′-(methylthio)-3,4′-bipyridine-3′,5′-dicarbnitrile (20)

A solution of **6** (0.5 mmole, 1.27 g) and iodomethane (1 mmole, 1.42 g) in methanol containing sodium methoxide (prepared by 0.6 g of sodium in 40 mL methanol) was stirred for 1 h. The product that formed was collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give **20** as pale yellow crystals, m.p. 280–282°C; **IR** (ν **cm**$^{-1}$): 3453, 3442, 3188 (NH_2); 2881–2923 (CH-aliphatic); 3066 (pyridine-CH) and 2217 (CN); **^{1}H-NMR** (**δ ppm**): 1.8 (s, 3H, CH_3), 4.8 (s, br., 2H, NH_2) and 7.3–8.6 (m, 4H, pyridine H's); **mass spectra** (**m/z, intensity %**) parent and base at m/z = 266 (100%) that lost SCH_2 fragment to give peak at m/z = 220 (7.1%) that lost a CN fragment to give peak at m/z = 194 (14.8%); Anal. for $C_{13}H_9N_5S$ (267.3): calcd./found (%): C, 58.41/58.4; H, 3.39/3.4; N, 26.20/26.3 S, 12.00/11.8.

Synthesis of 3-Amino-6-hydrazino-4-pyridin-3-yl-1H-pyrazolo-[3,4-b]-pyridine-5-carbonitrile (21)

A mixture of **6** (0.5 mmole, 1.27 g) or **20** (0.5 mmole, 1.34 g) and hydrazine hydrate (20–25 mL) was heated under reflux for 8–10 h until the odor of either H_2S or CH_3SH ceased. The product that formed after cooling was filtered off, washed with cold ethanol, and crystallized from ethanol to give **21**, as yellow crystals, m.p. 290–292°C; **IR** (**cm^{-1}**): 3462, 3287, 3187 (NH_2, NH), 3062 (pyridine-CH), 2987 (sat. CH) and 2213 (CN); **^{1}H-NMR** (**δ ppm**): 1.5 (s, 3H, CH_3), 3.7 (s, br., 4H, two NH_2), 5.4(s, br., 2H, two NH) and 7.5–8.6 (m, 4H, Pyridine H's); **mass spectra** (**m/z, intensity %**) parent and base peak at m/z = 266 (100%) that lost NH_2 to give peak at m/z = 250 (46.2%), which fragmented to give several peaks at low intensities; anal. for $C_{12}H_{10}N_8$ (266.2): calcd./found: C, 54.13/54.1, H, 3.79/3.8, N, 42.08/42.2.

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 green monkey kidney cells (Vero), a cell line obtained from American type culture collection (ATCC), and a human hepatoma cell line (Hep G2).

Viruses

Two models of DNA and RNA viruses were used for bioassay. These viruses were HSV-1 and Hepatitis A virus, 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 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 was used at 10% final concentration for the growth medium and at 2% for the maintenance medium.

Antibiotic-Antimycotic Mixture (GIBCO-BRL). Hundered X 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), 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 0.22 μm nitrocellulose membrane. The solution was used in washing cell monolayer sheets and in preparation of cell dissociation solution as follows.

Trypsin 1:250 (Sigma). Fifteen gram 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 2 mM solution (0.04 g) and was mixed with an equal volume of trypsin solution. The pH value of the trypsin-versene mixture was adjusted at 8.4 by 7.5% sodium bicarbonate solution and was 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 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 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' MEM. The MEM was supplemented with 10% FOBS and a 1% antibiotic-antimycotic mixture. The pH was adjusted at 7.2–7.4 by 7.5% sodium bicarbonate solution. The mixture was sterilized by filtration through a 0.22-μm pore-size nitrocellulose membrane.

III: Viruses

HSV-1 was obtained from the Environmental Virology Labortories, Department of Water Pollution Res., National Research Center (Dokki, Giza, 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 (10^5 cell/mL) and was incubated for 2 days at 37°C. HSV-1 was diluted to give 10^4 PFU/mL final concentration, and mixed with the plant extract at the previous concentration, and incubated overnight at 4°C. The growth medium was removed from the multiwell plate, and the virus-compound mixture was inoculated (100 μL/well). After 1 h of contact time, the inoculum was aspirated, and 3 mL of MEM with 1% agar rose was overlaid on 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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