Synthesis and Anti-Avian Influenza Virus (H5N1) Evaluation of Some Novel Nicotinonitriles and Their *N*-Acylic Nucleosides

Aymn E. Rashad,[a,b]* Ahmed H. Shamroukh,[a,c] Maher A. El-Hashash,[d] Ahmed F. El-Farargy,[e] Nabil M. Yousif,[a] Mowafia A. Salama,[a] Ahmed Mostafa,[f] and Mahmoud El-Shahat[a]

[a]Photochemistry Department, National Research Center, Dokki, Cairo, Egypt
[b]Chemistry Department, Faculty of Science and Human Studies, Horimlaa, Shaqra Univerisity, KSA
[c]Chemistry Department, Faculty of Science, Hail Univerisity, KSA
[d]Chemistry Department, Faculty of Science, Ain Shams Univerisity, Cairo, Egypt
[e]Chemistry Department Faculty of Science, Zagazig Univerisity, Zagazig, Egypt
[f]Virology Laboratory, Water Pollution Department, National Research Center, Dokki, Cairo, Egypt
*E-mail: aymnelzeny@yahoo.com

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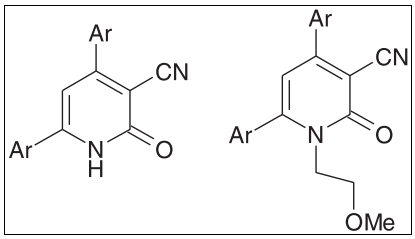

Pyridine and fused pyridines derivatives are interesting compounds with diverse chemical properties and pharmacological activities. Herein, the synthesis and antiviral evaluation of some new pyridines are described.

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INTRODUCTION

Pyridines and their analogs have been well recognized and documented in the literature as chemotherapeutic agents [1]. They are well known for their diverse therapeutic properties and exhibited antibacterial [2,3], anticancer [4,5], antiulcer [6], diuretics [7], anticonvulsant [8], antihypertensive [9], antitumor [10], antifungal [11,12], anti-AIDS [13], and antiviral [14] properties. On the other hand, the viral resistance to the used drugs is a major health problem and creates a pressing need for novel antiviral agents. Although, significant research is continuing in the area of antiviral drug discovery and there is still an urgent need to discover and develop new antiviral agents due to the increasing viral resistance to some commonly used drugs [15,16]. The manifold of diverse pharmacological activities shown by pyridine and its analogs and our interest in this area [17–20] led us to synthesize some new pyridin-2(1*H*)-ones/thione, tetrazolo-, triazolopyridines, and their *N*-acyclic nucleoside derivatives hoping to exhibit promising antiviral applications.

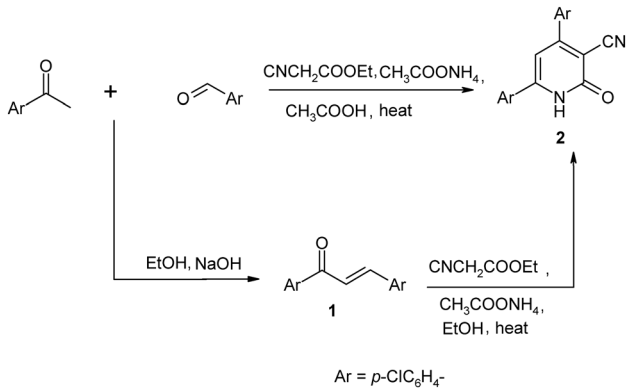
RESULTS AND DISCUSSION

In this work, enones are excellent starting materials for the synthesis of 4,6-bis(4-chlorophenyl)-2-oxo-1,2-dihydropyridine-3-carbinitrile (**2**) *via* the reaction of enone **1** [21] with ethyl cyanoacetate in the presence of ammonium acetate [22]. However, this procedure is time consuming and gives low yield. So, we report here an easy one-step synthesis which gives a higher yield of **2** by a four-component modified Hantzch reaction catalyzed by glacial acetic acid by heating a mixture of 4-chloroacetophenone, 4-benzaldehyde, ethyl cyanoacetate, ammonium acetate, and glacial acetic acid (Scheme 1). The elemental analysis and spectroscopic data are consistent with the assigned structure. IR spectrum of compound **2** indicated absorption bands due to NH, CN, and C═O groups and its ^{1}H NMR spectrum showed signals at δ 12.93 for NH, D_2O exchangeable. Also, its ^{13}C NMR spectrum revealed CN and C═O at δ 116.14 ppm and 161.97 ppm, respectively. Mass spectrum showed the molecular ion peak M^+ at *m/z* 340 as the base peak as well as the presence of isotopic pattern of two chlorine atoms.

Compound **2** was utilized as a key starting material in the synthesis of many novel heterocyclic compounds (Scheme 2). Thus, compound **2** was treated with phosphorus penta sulfide in xylene to afford the corresponding pyridinethione derivative **3**. The spectral data of compound **3** was in agreement with the proposed structure; in particular, the ^{13}C NMR spectrum which revealed C═S at δ 175.03 ppm (c.f.experimental).

When compound **2** was reacted with ethyl bromoacetate, in the presence of anhydrous potassium carbonate, it produced the ethyl ester derivative **4** (Scheme 2). The IR spectrum of the latter compound showed absorption bands at 2220 and 1742 cm^{-1} for CN and C═O ester, respectively. Its ^{1}H NMR spectrum showed

Scheme 1



signals at δ 1.16 (t, 3H, CH_3CH_2), 4.15 (q, 2H, CH_3CH_2), and 5.16 (s, 2H, OCH_2). Moreover, the absence of C═O signal in IR and ^{13}C NMR spectra of compound **4** revealed that the site of attack was on the O- and not N-atom [18].

When compound **4** was treated with hydrazine hydrate, in ethanol, the hydrazine derivative **6** was obtained and not the hydrazide derivative **5**. The IR and ^1H NMR spectra for compound **6** showed absorption bands for NH_2+ NH, CN, and the absence of C═O signals (c.f.experimental).

Due to the synthesis of acyclovir as one of the most potent antiviral drugs by Schaffer *et al*. [23], many attempts have been made by nucleoside chemists to prepare a number of related compounds with various side chains and glycons [23,24]. Thus, when the sodium salt of compound **2** (generated *in situ*) was treated with 2-chloroethyl methyl ether, chloroacetaldehyde dimethyl acetal, 2-chloroethanol, or 2-(2-chloroethoxy)ethanol, it afforded the corresponding *N*-acyclic nucleosides derivatives **7**–**10**, respectively (Scheme 2).

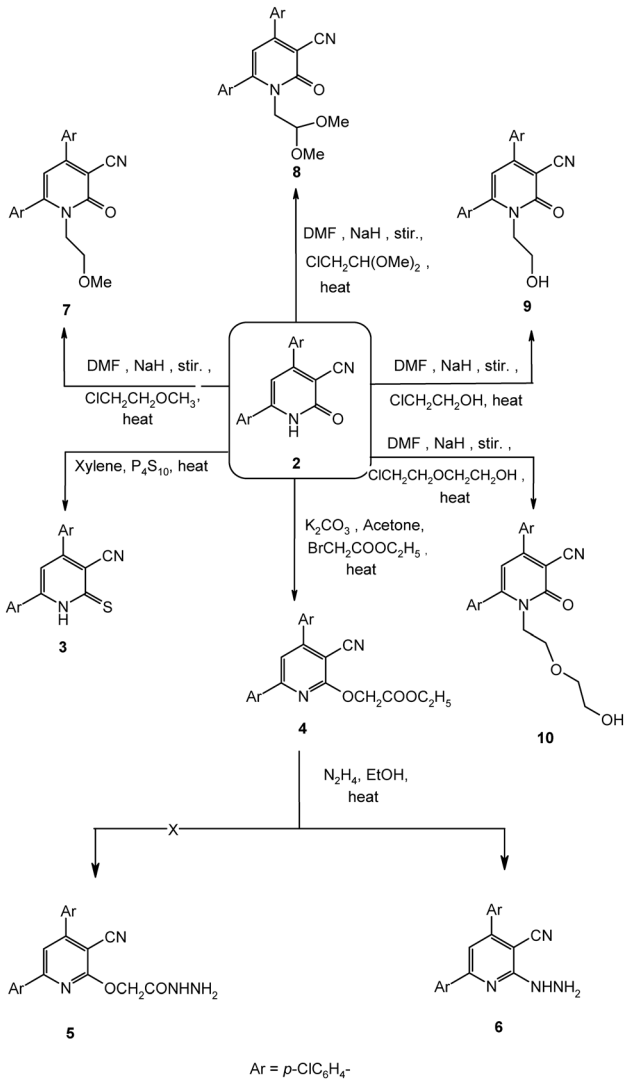
The structure of the aforementioned acyclic nucleosides was confirmed with spectral data and the NMR spectra revealed methoxyethyl, dimethoxyethyl, hydroxyethyl, and hydroxylethoxyethyl signals. In addition, the IR and ^{13}C NMR spectra revealed that the site of attack was on the N- and not O-atom. This is due to the fact that the nitrogen atom behaves as a nucleophile which attacks an electrophilic carbon of an alkyl halide [17].

Nitrozation of compound **6** gave the corresponding 5,7-bis(4-chlorophenyl)-tetrazolo[1,5-*a*]pyridine-8-carbonitrile (**11**). The IR and ^1H NMR spectra of **11** revealed the absence of NH_2 and NH groups and its mass spectrum showed the molecular ion peak M^+ at *m/z* 365 as the base peak (c.f. experimental).

Synthesis of 2-amino-4,6-bis(4-chlorophenyl)nicotinonitrile **12** was achieved when compound **11** was heated with zinc dust in glacial acetic acid (c.f. experimental). The IR spectrum of **12** showed bands for NH_2 and CN groups and its ^1H NMR spectrum showed signals at 7.08 (s, 2H, NH_2, D_2O exchangeable).

On the other hand, when compound **6** was refluxed with acetic anhydride or trimethyl orthoformate, it afforded

Scheme 2



[1,2,4]triazolo[4,3-*a*]pyridine-8-carbonitriles **13** and **14**, respectively (Scheme 3). The structure of the new products was established according to their elemental and spectroscopic data. The IR spectrum of **13** and **14** revealed the absence of NH_2, NH groups, and its ^1H NMR spectrum showed signals for CH_3 and triazol-H3.

Condensation of compound **6** with acetyl acetone, gave 4,6-bis (4-chlorophenyl)-2-(3,5-dimethyl-1*H*-pyrazol-1-yl)-nicotinonitrile (**15**). The IR spectrum of **15** showed bands at 2210 cm^{-1} of CN and its ^1H NMR spectrum showed signals at δ 2.3, 2.5 for two CH_3 and at 6.1 (s, 1H, pyrazole-H). Also, its mass spectrum showed the molecular ion peak M^+ at *m/z* 418 as the base peak.

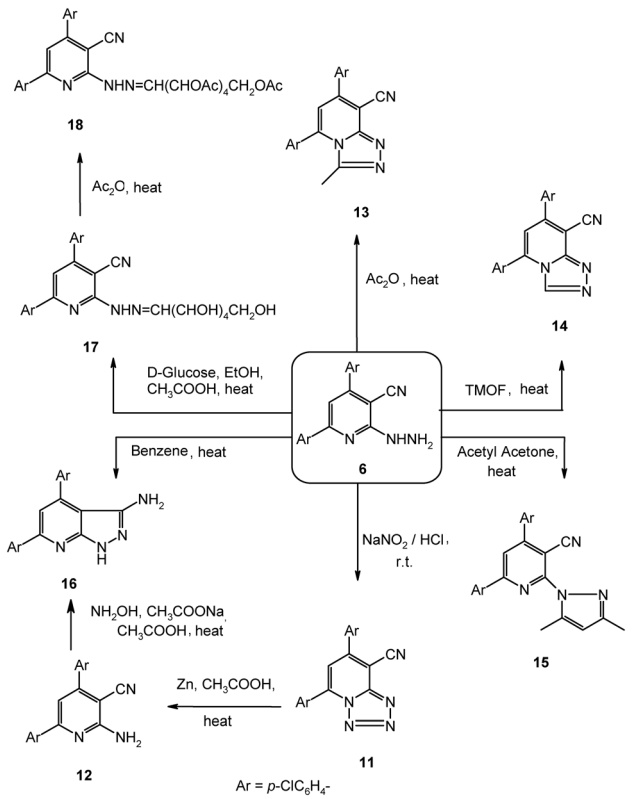
Moreover, refluxing of compound **6** in dry benzene afforded 4,6-bis(4-chlorophenyl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-amine (**16**); which was also obtained by heating compound **12** with hydroxyl amine in the presence of sodium acetate in

a high yield [25]. The IR and ^{1}H NMR spectra of compound **16** showed bands characterized for NH and NH_2.

On the other hand, the 4,6-bis(4-chlorophenyl)-2-(2,3,4,5,6-pentahydroxyhexylidene) hydrazinyl)-nicotinonitriles (**17**) was prepared by heating compound **6** with D-glucose (Scheme 3). The formed product revealed absorption frequencies due to OH, NH, CN, and C═N in IR spectrum and its ^{1}H NMR spectrum showed the presence of the sugar protons, NH, and azomethine (CH═N) [26]. Acetylation of the hydrazone derivative **17** by heating in acetic anhydride gave its corresponding *O*-acetylated sugar hydrazone derivative **18**. The IR spectrum of the latter compound revealed the absence of hydroxyl groups and showed absorption bands due to NH and C═O groups, whereas its ^{1}H NMR spectrum showed the presence of OAc and NH signals (c.f.experimental).

Antiviral bioassays. Antiviral bioassays were carried out to test compounds **2**–**4**, **6**–**18**. The concentrations of the tested compounds which exhibited 50% cytotoxicity (LD50) and the 50% effective antiviral concentration (EC50) were determined in addition to the cytotoxicity (TC) and the therapeutic index (TI) (Table 1). It was obvious that, at different concentrations (5, 10, 20, and 40 μg), compounds **2**, **3**, **6**, **10**, **12**, and **16** showed higher therapeutic indices than the other tested compounds and the results were compared with the anti-influenza drug zanamivir.

Scheme 3



CONCLUSIONS

Structural activity correlations of the obtained results indicated that, replacement of the C═O group in compound **2** with thio (compound **3**), alkoxy (compound **4**), hydrazino group (compound **6**), or substituted with acyclic sugar chain (compounds **7**–**10**) decreased the antiviral activity. While, the presence of pyrazolyl ring (compound **15**) and side chain of acyclic sugar hydrazone (compounds **17** and **18**) increased the H5N1 activity but not as much as that of compound **2** (Fig. 1).

EXPERIMENTAL

All melting points are uncorrected and measured using Electro-Thermal IA 9100 apparatus (Shimadzu, Japan). Infrared spectra were recorded as potassium bromide pellets on a Perkin-Elmer 1650 spectrophotometer, National Research Center, Cairo, Egypt. ^{1}H NMR and ^{13}C NMR spectra were determined on a Jeol-Ex-500 NMR spectrometer and chemical shifts were expressed as part per million; (δ values, ppm) against TMS as internal reference, National Research Center, Cairo, Egypt. Mass spectra were recorded on EI + Q1 MSLMR UPLR, National Research Center, Cairo, Egypt. Microanalyses were operated using Mario Elmentar apparatus, Organic Microanalysis Unit, National Research Center, Cairo, Egypt. Column Chromatography was performed on (Merck) Silica gel 60 (particle size 0.06–0.20 mm).

4,6-Bis(4-chlorophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (2). ***Method A.*** A mixture of compound 1 [21] (0.01 mole), ethyl cyanoacetate (0.01 mole), and ammonium acetate (0.08 mole) in ethanol (40 mL) was refluxed for 10 h. After cooling, the precipitate was filtered off, dried, and recrystallized from glacial acetic acid to give 2.

Method B. A mixture of 4-chloroacetophenone (0.01 mole), 4-chlorobenzaldehyde (0.01 mole), ethyl cyanoacetate (0.01 mole), and ammonium acetate (0.08 mole) in glacial acetic acid (40 mL) was refluxed for 3 h. After cooling, the precipitate was

Table 1

Antiviral activity against H5N1 virus of the prepared compounds by determination of both EC50 and LD50.

Compound	TC50 (μg/mL)	Avian influenza virus (H5N1) IC_{50} (μg/mL)	Therapeutic index (TI)
2	215.25	152.66	1.41
3	168.9	178.26	0.948
4	138.18	153.54	0.9
6	133.38	99.54	1.34
7	151.15	160.8	0.94
8	176.12	238	0.74
9	13.30	134.44	0.099
10	111.1	113.4	0.98
11	133.3	156.94	0.85
12	111.19	84.24	1.32
13	133	137.16	0.97
14	169.19	176.24	0.96
15	174.59	185.74	0.94
16	155.19	156.76	0.99
17	158	164.62	0.96
18	141.3	153.6	0.92
Zanamivir	76	5	15.2

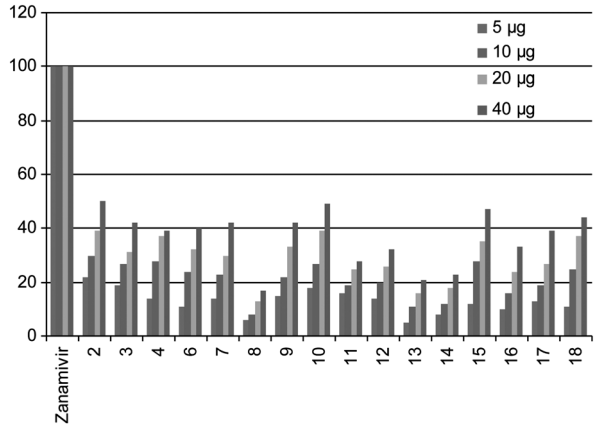


Figure 1. Antiviral activity of compounds **2**–**4**, **6**–**18** at different concentrations.

filtered off, dried, and recrystallized from glacial acetic acid to give 2. Yield (54% from method A and 91% from method B), mp 305–306°C. IR (KBr) v, cm^{-1}: 3279 (NH), 2215 (CN), and 1629 (C═O). ^1H NMR spectrum (DMSO-d_6, δ ppm): 6.88 (s, 1H, pyridine-H5), 7.57–7.90 (m, 8H, Ar-H), 12.87 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6, δ ppm): 116.14 (CN), 128.77–150.60 (16 Ar-C), 161.97 (C═O). Ms: m/z (344, $4M^+$, 9%), (342, $2M^+$, 51%), (340, M^+, 100%). Anal. Calcd. for $C_{18}H_{10}Cl_2N_2O$ (341.20): C, 63.36; H, 2.95; Cl, 20.78; N, 8.21; found C, 63.27; H, 2.88; Cl, 20.71; N, 8.16.

4,6-Bis(4-chlorophenyl)-2-thioxo-1,2-dihydropyridine-3-carbonitrile (3). To a solution of compound 2 (0.01 mole) in dry xylene (50 mL), phosphorus pent sulfide (0.04 mol) was added and the reaction mixture was heated under reflux for 2 h. Then the excess solvent was evaporated under reduced pressure and the residue was recrystallized from dioxane to give 3. Yield 37%, mp 237–238°C. IR (KBr) v, cm^{-1}: 3223 (NH), 2216 (CN), 1272 (C═S). The ^1H NMR spectrum (DMSO-d_6, δ ppm): 7.42–8.33 (m, 10H, 8Ar-H + pyridine-H5 + NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6, δ ppm): 120.70 (CN), 128.34–159.22 (16 Ar-C), 175.03 (C═S). Ms: m/z (360, $4M^+$, 7%), (358, $2M^+$, 42%), (356, M^+, 85%). Anal. Calcd. for $C_{18}H_{10}Cl_2N_2S$ (357.26): C, 60.52; H, 2.82; Cl, 19.85; N, 7.84; S, 8.97; found C, 60.44; H, 2.77; Cl, 19.79; N, 7.80; S, 8.88.

Ethyl 2-(4,6-bis(chlorophenyl)-3-cyanopyridin-2-yloxy)acetate (4). A mixture of compound 2 (0.01 mole), ethyl bromoacetate (0.01 mole), and anhydrous potassium carbonate (0.04 mole) in dry acetone (30 mL) was refluxed for 20 h. The excess solvent was evaporated under reduced pressure and the solid obtained was recrystallized from ethanol to give 4. Yield 83%, mp 187–188°C. IR (KBr) v, cm^{-1}: 2220 (CN), 1742 (C═O). The ^1H NMR spectrum (DMSO-d_6, δ ppm): 1.16 (t, J = 8 Hz, 3H, CH_3CH_2O), 4.20 (q, J = 8 Hz, 2H, CH_3CH_2O), 5.16 (s, 2H, CH_2), 7.58–8.20 (m, 9H, 8Ar-H + pyridine-H5). ^{13}C NMR spectrum (DMSO-d_6, δ ppm): 14.99 (CH_3), 61.23 (CH_2), 64.26 (OCH_2CO), 115.00 (CN), 129.38–136.34 (16 Ar-C), 168.62 (C═O). Ms: m/z (430, $4M^+$, 11%), (428, $2M^+$, 45%), (426, M^+, 100%). Anal. Calcd. for $C_{22}H_{16}Cl_2N_2O_3$ (427.29): C, 61.84; H, 3.77; Cl, 16.59; N, 6.56; found C, 61.79; H, 3.70; Cl, 16.53; N, 6.46.

4,6-Bis(4-chlorophenyl)-2-hydrazinylnicotinonitrile (6). A solution of compound 4 (0.01 mole) in ethanol (50 mL) and hydrazine hydrate (0.02 mole, 99%) was refluxed for 6 h. After cooling, the separated solid was collected and recrystallized from ethanol to give 6. Yield 77%, mp178–179°C. IR (KBr) v, cm^{-1}: 3398–3322 (NH_2, NH), 2203 (CN). ^1H NMR spectrum (DMSO-d_6, δ ppm): 4.61 (bs, 2H, NH_2, D_2O exchangeable), 7.37–8.33 (m, 9H, 8Ar-H + pyridine-H5), 8.49 (s, 1H, NH, D_2O exchangeable). Ms: m/z (358, $4M^+$, 3%), (356, $2M^+$, 81%), (354, M^+, 100%). Anal. Calcd. for $C_{18}H_{12}Cl_2N_4$ (355.23): C, 60.86; H, 3.41; Cl, 19.96; N, 15.77; found C, 60.81; H, 3.36; Cl, 19.87; N, 15.72.

General procedure for synthesis of 7–10. To a solution of compound 2 (0.01 mole) in dry DMF (50 mL), 50% oil-immersed sodium hydride (0.20 g) was added. Thereafter, the reaction mixture was stirred at room temperature for 1 h. Then, 2-chloroethyl methyl ether, chloroacetaldehyde dimethylacetal, 2-chloroethanol or 2-(2-chloroethoxy)ethanol (0.02 mol) was added and the reaction mixtures were stirred at 70°C for 3 h, 5 h, 4 h, 3 h, and 2 h, respectively. After evaporation under reduced pressure, the residues were purified on silica gel column using chloroform: methanol (9:1) as an eluent to give compounds 7, 8, 9, and 10, respectively.

4,6-Bis(4-chlorophenyl)-1-(2-methoxyethyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (7). Yield 68%, mp 153–154°C. IR (KBr) v, cm^{-1}: 2218 (CN), 1673 (C═O). ^1H NMR spectrum ($CDCl_3$, δ ppm): 3.48 (s, 3H, OCH_3), 3.85 (t, J = 4.6 Hz, 2H, NCH_2), 4.72 (t, J = 3.8 Hz, 2H, CH_2O), 7.39 (s, 1H, pyridine-H5), 7.44–8.00 (m, 8H, Ar-H). ^{13}C NMR spectrum ($CDCl_3$, δ ppm): 30.43 (NCH_2), 59.49 (OCH_3), 70.50 (CH_2O), 115.11 (CN), 128.68–136.95 (16 Ar-C), 164.60 (C═O). Anal. Calcd. for $C_{21}H_{16}Cl_2N_2O_2$ (399.28): C, 63.17; H, 4.04; Cl, 17.76; N, 7.02; found C, 63.09; H, 3.95; Cl, 17.70; N, 6.95.

4,6-Bis(4-chlorophenyl)-1-(2,2-dimethoxyethyl)2-oxo-1,2-dihydropyridine-3-carbonitrile (8). Yield 53%, mp 171–172°C. IR (KBr) v, cm^{-1}: 2220 (CN), 1671 (C═O). ^1H NMR spectrum ($CDCl_3$, δ ppm): 3.51(s, 6H, $2OCH_3$), 4.60 (d, J = 4.55 Hz, 2H, NCH_2), 4.84 (t, J = 7.3 Hz, 1H, CH), 7.41 (s, 1H, pyridine-H5), 7.45–8.00 (m, 8H, Ar-H). Anal. Calcd. for $C_{22}H_{18}Cl_2N_2O_3$ (429.31): C, 61.55; H, 4.23; Cl, 16.52; N, 6.53; found C, 61.49; H, 4.17; Cl, 16.46; N, 6.45.

4,6-Bis(4-chlorophenyl)-1-(2-hydroxyethyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (9). Yield 59%, mp 183–184°C. IR (KBr) v, cm^{-1}: 3400 (OH), 2211 (CN), 1678 (C═O). ^1H NMR spectrum (DMSO-d_6, δ ppm): 3.79 (t, J = 4.50 Hz, 2H, NCH_2), 4.58 (t, J = 4.55 Hz, 2H, CH_2O), 5.43 (bs, 1H, OH, D_2O exchangeable), 7.50–8.22 (m, 9H, 8Ar-H+ pyridine-H5). Anal. Calcd. for $C_{20}H_{14}Cl_2N_2O_2$ (385.25): C, 62.35; H, 3.66; Cl, 18.41; N, 7.27; found C, 62.27; H, 3.58; Cl, 18.32; N, 7.18.

4,6-Bis(4-chlorophenyl)-1-(2-(2-hydroxyethoxy)ethyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (10). Yield 54%, mp 191–192°C. IR (KBr) v, cm^{-1}: 3340 (OH), 2216 (CN), 1670 (C═O). ^1H NMR spectrum ($CDCl_3$, δ ppm): 2.10 (t, J = 4.7 Hz, 2H, NCH_2), 2.91 (t, J = 4.49 Hz, 2H, CH_2O), 3.6 (m, 4H, OCH_2CH_2OH), 5.0 (bs, OH, D_2O exchangeable), 7.50–8.22 (m, 9H, 8Ar-H+ pyridine-H5). Anal. Calcd. for $C_{22}H_{18}Cl_2N_2O_3$ (429.31): C, 61.55; H, 4.23; Cl, 16.52; N, 6.53; found C, 61.47; H, 4.19; Cl, 16.42; N, 6.45.

5,7-Bis(4-chlorophenyl)-tetrazolo[1,5-a]pyridine-8-carbonitrile (11). To an ice-cold solution of compound 6 (0.01 mole) in glacial acetic acid (10 mL), a solution of sodium nitrite [prepared by dissolving sodium nitrite (0.01 mole) in water (3 mL)] was added drowsily in an ice bath. The reaction mixture was allowed to stand overnight at room temperature and then was poured into water. The formed solid was filtered off, washed with water, dried, and recrystallized from ethanol to give 11. Yield 60%, mp 157–158°C. IR (KBr) v, cm^{-1}: 2213 (CN). ^1H NMR spectrum (DMSO-d_6, δ ppm): 7.64–8.41 (m, 9H, 8Ar-H+pyridine-H5). Ms: m/z (369, $4M^+$, 10%), (367, $2M^+$, 61%), (365, M^+, 100%). Anal. Calcd. for $C_{18}H_9Cl_2N_5$ (366.21): C, 59.04; H, 2.48; Cl, 19.36; N, 19.12; found C, 59.00; H, 2.39; Cl, 19.28; N, 19.05.

2-Amino-4,6-bis(4-chlorophenyl)-nicotinonitrile (12). A mixture of compound 11 (0.01 mole), zinc dust (0.01 mole), and glacial acetic acid (10 mL) was stirred at room temperature for 2 h and

then was heated on water bath at 80°C for 8 h. The reaction mixture was poured into water, extracted with chloroform, and the extract was evaporated under reduced pressure. The formed solid was recrystallized from ethanol to give 12. Yield 40%, mp 193–194°C. IR (KBr) v, cm^{-1}: 3392 (NH_2), 2205 (CN). ^1H NMR spectrum (DMSO-d_6, δ ppm): 7.08 (bs, 2H, NH_2, D_2O exchangeable), 7.28 (s, 1H, pyridine-H5) 7.52–814 (m, 8H, Ar-H). Ms: m/z (343, $4M^+$, 11%), (341, $2M^+$, 70%), (339, M^+, 100%). Anal. Calcd. for $C_{18}H_{11}Cl_2N_3$ (340.21): C, 63.55; H, 3.26; Cl, 20.84; N, 12.35; found C, 63.48; H, 3.20; Cl, 20.79; N, 12.26.

5,7-Bis(4-chlorophenyl)-3-methyl-[1,2,4]triazolo[4,3-a] pyridine-8-carbonitrile (13). A mixture of compound 5 (0.01 mole) and acetic anhydride (10 mL) was refluxed for 6 h. The reaction mixture was cooled and poured into cold water. The separated solid was filtered off, dried, and recrystallized from ethanol to give 13. Yield 82%, mp 174–175°C. IR (KBr) v, cm^{-1}: 2210 (CN). ^1H NMR spectrum ($CDCl_3$, δ ppm): 2.21 (s, 3H, CH_3), 7.64–8.41 (m, 9H, 8Ar-H + pyridine-H5). Ms: m/z (382, $4M^+$, 3%) (380, $2M^+$, 34%), (378, M^+, 78%), (363, M^+-CH_3, 100%). Anal. Calcd. for $C_{20}H_{12}Cl_2N_4$ (379.25): C, 63.34; H, 3.19; Cl, 18.70; N, 14.77; found C, 63.24; H, 3.11; Cl, 18.62; N, 14.68.

5,7-Bis(4-chlorophenyl)-[1,2,4]triazolo[4,3-a]pyridine-8-carbonitrile (14). A mixture of compound 6 (0.01 mole) and trimethyl orthoformate (20 mL) was refluxed for 15 h. The reaction mixture was filtered off on hot and the separated solid was recrystallized from ethanol to give 14. Yield 79%, mp 279–280°C. IR (KBr) v, cm^{-1}: 2211(CN). ^1H NMR spectrum (DMSO-d_6, δ ppm): 7.77–8.41 (m, 10H, 8Ar-H + pyridine-H5+ triazol-H3). Ms: m/z (368, $4M^+$, 4%), (366, $2M^+$, 53%), (364, M^+, 100%). Anal. Calcd. for $C_{19}H_{10}Cl_2N_4$ (365.22): C, 62.49; H, 2.76; Cl, 19.41; N, 15.34; found C, 62.39; H, 2.71; Cl, 19.35; N, 15.26.

4,6-Bis(4-chlorophenyl)-2-(3,5-dimethyl-1H-pyrazol-1-yl) nicotinonitrile (15). A mixture of compound 6 (0.01 mole) and acetyl acetone (0.02 mole) in ethanol (20 mL) was refluxed for 8 h. After cooling, the separated solid was filtered off, dried, and recrystallized from ethanol to give 15. Yield 79%, mp 192–193°C. IR (KBr) v, cm^{-1}: 2210 (CN). ^1H NMR spectrum ($CDCl_3$, δ ppm): 2.35 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 6.11 (s, 1H, pyrazole) 7.26–8.08 (m, 9H, 8Ar-H + pyridine-H5). Ms: m/z (422, $4M^+$, 14%), (420, $2M^+$, 67%), (418, M^+, 100%). Anal. Calcd. for $C_{23}H_{16}Cl_2N_4$ (419.32): C, 65.88; H, 3.85; Cl,16.91; N, 13.36; found C, 65.79; H, 3.75; Cl, 16.82; N, 13.30.

4,6-Bis(4-chlorophenyl)-1H-pyrazolo[3,4-b]pyridin-3-amine (16). ***Method A.*** Compound 6 (0.01 mole) in dry benzene (20 mL) was refluxed for 5 h. After cooling, the separated solid was filtered off, dried, and recrystallized from dioxane to give 16.

Method B. A mixture of compound 12 (0.01 mole), hydroxylamine hydrochloride (0.02 mole), sodium acetate (0.01 mole), and glacial acetic acid (15 mL) was refluxed for 7 h; left overnight at room temperature, and then poured into cold water. The solid that precipitated was filtered off, dried, and recrystallized from dioxane to give 16. Yield (39% from method A, 73 % from method B), mp 257–258°C. IR (KBr) v, cm^{-1}: 3340–3320 (NH+NH_2). ^1H NMR spectrum (DMSO-d_6, δ ppm): 4.61 (bs, 2H, NH_2, D_2O exchangeable), 7.77–9.21 (m, 9H, 8Ar-H+ pyridine-H5), 9.32 (s, 1H, NH, D_2O exchangeable). Ms: m/z (358, $4M^+$, 12%), (356, $2M^+$, 61%), (354, M^+, 100%). Anal. Calcd. for $C_{18}H_{12}Cl_2N_4$ (355.23): C, 60.86; H, 3.41; Cl, 19.96; N, 15.77; found C, 60.80; H, 3.33; Cl, 19.87; N, 15.70.

4,6-Bis(4-chlorophenyl)-2-(2,3,4,5,6-pentahydroxyhexylidene) hydrazinyl)- nicotinonitrile (17). A mixture of compound 6 (0.01 mole), D-glucose (0.01 mole) in ethanol (30 mL) and a catalytic amount of glacial acetic acid (three drops) was heated at 80°C for 2 h. The formed precipitate was filtered off, washed with water several times, and dried to give 17. Yield 47%, mp 147–149°C. IR (KBr) v, cm^{-1}: 3350–3260 (OH + NH), 2211 (CN). The ^1H NMR spectrum (DMSO-d_6, δ ppm): 2.90–3.37 (protons of the alditol congregated with the solvent absorption) [23], 3.38–3.40 (m, 2H, CH_2OH), 4.33–4.86 (m, 5H, 5OH, D_2O exchangeable), 6.15 (s,1H, NH, D_2O exchangeable), 7.42–8.10 (m, 9H, 8Ar-H +pyridine-H5), 8.30 (s, 1H, CH═N). ^{13}C NMR spectrum ($CDCl_3$, δ ppm): 61.73–73.59 (5C-alditol), 115.11 (CN), 128.68–136.95 (17Ar-C+N═CH). Anal. Calcd. for $C_{24}H_{22}Cl_2N_4O_5$ (517.37): C, 55.72; H, 4.29; Cl, 13.71; N, 10.83; found C, 55.65; H, 4.22; Cl, 13.61; N, 10.74.

6-(2-[4,6-Bis(4-chlorophenyl)-3-cyanopyridin-2-yl]hydrazono) hexane-1,2,3,4,5-pentaylpentaacetate (18). Compound 17 (0.01 mole) in acetic anhydride (20 mL) was refluxed on water bath for 3 h. The reaction mixture was poured onto ice water with stirring and the solid that precipitated was collected by filtration, washed with water, dried, and purified using silica gel column using petroleum ether:ethyl acetate (8:2) as an eluent to give 18. Yield 63%, mp 189–191°C. IR (KBr) v, cm^{-1}: 3230 (NH), 2220 (CN), 1730 (C═O). The ^1H NMR spectrum (DMSO-d_6, δ ppm): 2.01–2.11 (5s, 15H, 5OAc), 3.02–3.05 (m, 2H, CH_2OAc), 3.86–3.88 (m, 1H, CHOAc), 4.06–4.12 ((m, 2H, CH_2OAc), 5.10–5.15 (m, 1H, CHOAc), 5.70–7.73 (m, 1H, CHOAc), 7.46–8.00 (m, 9H, 8Ar-H+pyridine-H5), 8.02 (s, 1H, CH═N), 11.05 (s, 1H, NH, D_2O exchangeable). Anal. Calcd. for $C_{34}H_{32}Cl_2N_4O_{10}$ (727.56): C, 56.13; H, 4.43; Cl, 9.75; N, 7.70; found C, 56.04; H, 4.35; Cl, 9.68; N, 7.60.

Antiviral bioassay. ***Virus and cells.*** Avian influenza A virus (H5N1), isolated from Egypt in 2006, was used in this study to evaluate antiviral activity of some synthetic compounds. Madin-Darby canine kidney (MDCK) cells used for virus propagation were friendly obtained from St. Jude Children's Research Hospital. The MDCK cells were routinely passaged in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic mixture (penicillin-streptomycin-amphotericin B).

MTT assay (cytotoxicity assay). The stock samples were diluted with Dulbecco's Modified Eagle's Medium (DMEM) to desired concentrations. Stock solutions of the test compounds were prepared in DMSO at a concentration of 10% in ddH_2O. The cytotoxic activity of the extracts were tested in MDCK cell line by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mossman, 1983) [27] with minor modification. Briefly, the cells were seeded in 96-well plates (100 μL/well at a density of 3×10^5 cells/mL) and treated with various concentrations of the sample solutions. At 24 h, cells were washed with sterile phosphate buffer (PBS) three times and the supernatant was discarded. MTT solution (20 μL of 5 mg/mL) was added to each well and incubated at 37°C for 4 h.

$$\% \text{ Cytotoxicity} = \frac{(\text{Absorbance of cell without treatment} - \text{Absorbance of cell with treatment}) \times 100}{\text{Absorbance of cell without treatment}}.$$

Then, the medium was aspirated, and in each well, the formed formazan crystals were dissolved with 200 μL of acidified isopropanol (0.04*M* HCl in absolute isopropanol). An absorbance of formazan was detected by a dual wavelength UV spectrometer at 540 nm with 620 nm reference wavelength. The percentage of cytotoxicity compared with the untreated cells was determined with the equation given above. The plot of % cytotoxicity *versus* sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (TC50).

Plaque reduction assay. In a six-well plate, confluent MDCK cells were infected with a preincubated mixture of 100 mL of avian influenza H5N1 virus (80–100 plaques/well), 100 mL of DMEM [containing 2% antibiotics and 1 mg/mL of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TCPK)], and different concentration of each compound (5, 10, 20, and 40 mg/mL). The plates were incubated for 45 min at 37°C in 5% CO_2 to allow virus adsorption. After adsorption, 2 mL of agarose overlayer in 2× DMEM containing 1% FBS was added to each well and mixed. The cultures were incubated at 37°C in 5% CO_2 for 3–4 days. Plaques were fixed with 3.7% formalin in phosphate-buffered saline for 2 h followed by removal of the agar overlayer and staining with 0.10% crystal violet in distilled water. Plaques were counted manually from triplicate wells based on plaque number but not plaque size. Viral counts and percentage of virus reduction were calculated according to Hayden *et al*. [28]. The plot of % of reduction *versus* sample concentration was used to calculate the concentration which exhibited 50% inhibition of viral plaques (IC50). The therapeutic index is calculated by dividing TC_{50} on IC_{50}.

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