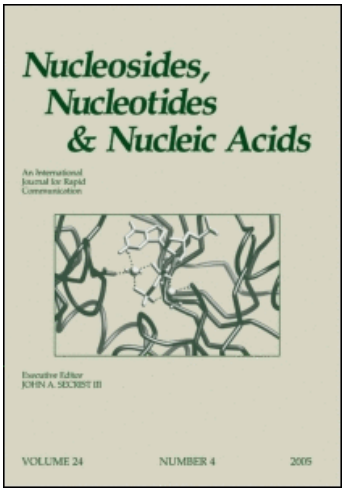

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A Facile Synthesis and Anti-Avian Influenza Virus (H5N1) Screening of Some Novel Pyrazolopyrimidine Nucleoside Derivatives

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A FACILE SYNTHESIS AND ANTI-AVIAN INFLUENZA VIRUS (H5N1) SCREENING OF SOME NOVEL PYRAZOLOPYRIMIDINE NUCLEOSIDE DERIVATIVES

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□ *Treatment of 5-amino-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-d]pyrimidin-11-yl)-1H-pyrazole-4-carbonitrile (**1**) with formic acid afforded pyrazolo[3,4-d]pyrimidin-4-one derivative **2**. The sodium salt of the latter compound (generated in situ) was treated with some alkyl halides to afford the corresponding N-substituted compounds **3**–**7**. The siloxy derivative **8** (generated also in situ from **2**) was ribosylated and glycosylated to yield compounds **9** and **11**, respectively. Deprotection of compounds **9** and **11** in methanolic ammonia produced the free nucleosides **10** and **12**, respectively. Moreover, the prepared compounds were tested for antiviral activity against H5N1 virus [A/chicken/Egypt/1/2006] and some of them revealed moderate results compared with the other tested compounds.*

Keywords Pyrazoles; pyrazolopyrimidines; nucleosides; anti-avian influenza virus (H5N1)

INTRODUCTION

In recent years, the incidence of avian viral infection has increased over the world as a result of virus resistance.[1,2] However, transmission of Influenza A (H5N1) virus between humans is very limited at present, but continued monitoring is required to identify any increase in viral adaptation to human hosts.[3,4] This problem demands to find out new antiviral compounds to defeat virus that is increasingly resistant to drugs.

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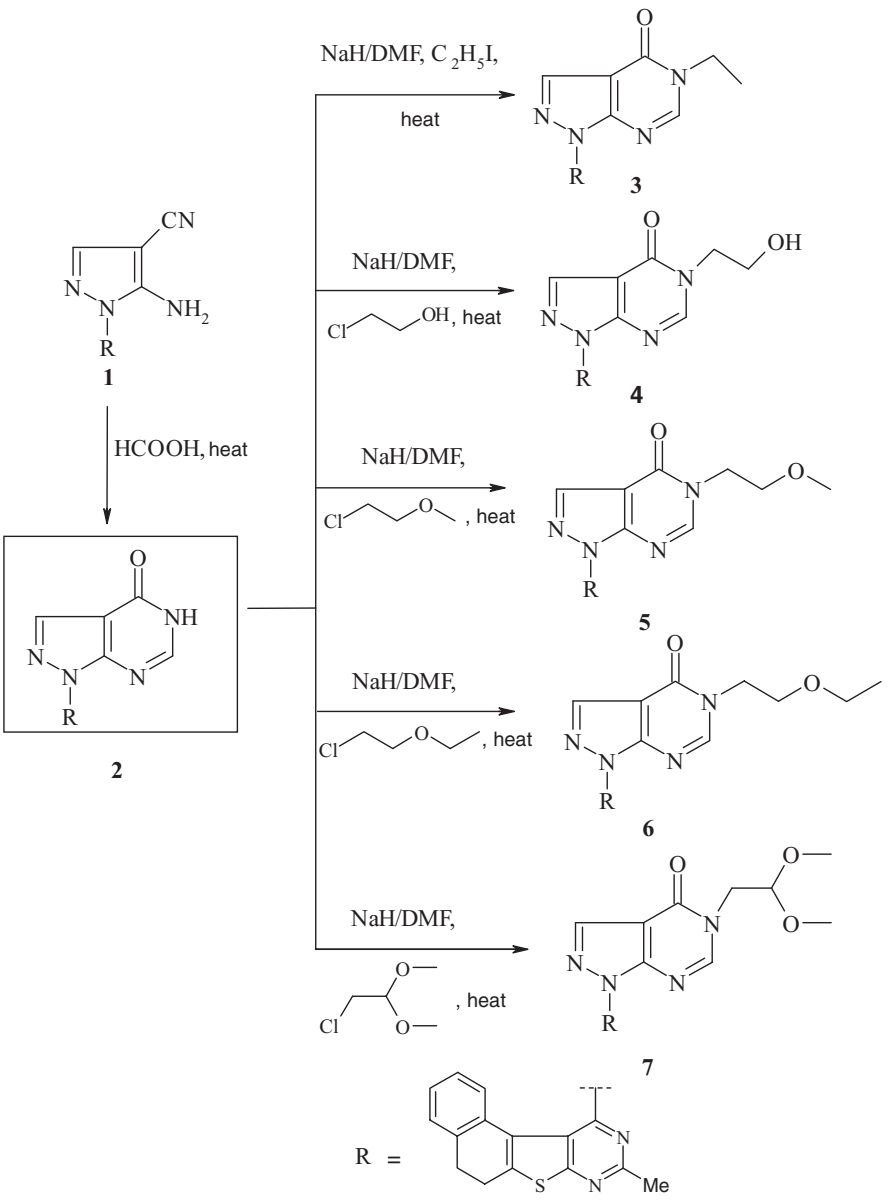
Moreover, pyrazole and pyrazolopyrimidine derivatives are well established in the literature as important biologically active heterocyclic compounds.[5–8] These derivatives are the subject of many research studies due to their widespread potential biological activities such as anti-inflammatory,[9] antimicrobial,[10] antitumor,[11] and antiviral agents.[12,13] Until now, the binding of heterocyclic compounds with acyclic and cyclic sugar moiety forming the corresponding nucleosides has commanded the worldwide attention of many research groups because of their high potential to exhibit chemotherapeutic activity.[14,15] In this context, the study of many synthetic heterocyclic nucleosides as potent antivirals has generated much interest in recent years, and many open-chain carbohydrate residues have been reported and evaluated as potential antiviral drugs.[16–18] Based on the above mentioned research results, the goal of this study is to synthesize novel pyrazolopyrimidine derivatives with acyclic and cyclic substituents for the purpose of enhancing the expected biological effects as antiviral agents.

RESULTS AND DISCUSSION

Thus, treatment of 1-substituted 5-aminopyrazole-4-carbonitrile **1**[19] with formic acid afforded pyrazolo[3,4-*d*]pyrimidin-4-one derivative **2** in a quantitative yield as key compound for further synthesis. The structure of the latter compound was confirmed on the basis of its elemental analysis and spectral data (see Experimental, Scheme 1). In particular, the infrared (IR) spectrum of compound **2** showed absorption bands assignable to the NH, C=O as well as the absence of CN group and MS gave the molecular ion peak at m/z (%): 386 [M^+, 84].

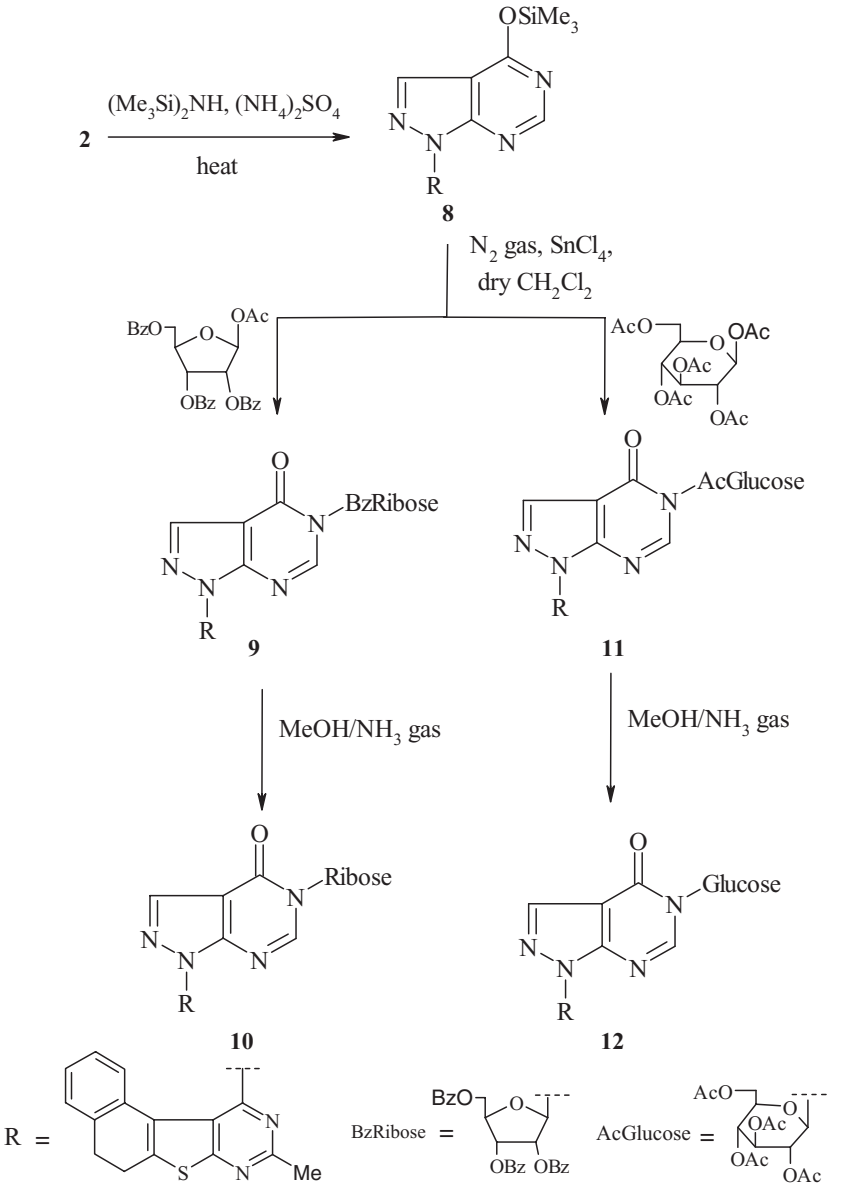
The sodium salt of derivative **2** was treated with ethyl iodide, 2-chloroethanol, 2-chloroethyl methyl ether, 2-chloroethyl ethyl ether, or 2-chloroacetaldehyde dimethylacetal to afford compounds **3–7**, respectively (Scheme 1). The IR and ^{13}C NMR spectra of the latter compounds revealed the appearance of the C=O signal and the ^1H NMR spectra indicated the absence of the NH group and the presence of ethyl, hydroxyethyl, methoxyethyl, ethoxyethyl, and dimethoxyethyl signals, respectively (see Experimental). This due to the fact that the nitrogen atom behaves as a nucleophile attacks an electrophilic carbon atom of an alkyl halide.

Heating of compound **2** with hexamethyldisilazane (HMDS) in the presence of ammonium sulfate gave the silyloxypyrimidine derivative **8**, which was subsequently treated with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in the presence of $SnCl_4$ according to the method of Niedballa and Vorbrüggen[20] to afford the corresponding N-riboside **9** (Scheme 2). The ^1H NMR spectrum of compound **9** showed a doublet at δ 6.69 ppm ($J_{1',2'}$ = 4.40 Hz) assigned to the anomeric proton indicating the β-configuration[21] and the other sugar protons resonate at δ 4.10–4.88 ppm.



SCHEME 1 Synthesis routes of compounds 2–7.

Debenzoylation of the blocked riboside **9** was achieved in methanolic ammonia at 0°C to afford the desired free 1-β-D-ribofuranosyl derivative **10**. The IR and ^{1}H NMR spectrum of the latter compound showed the presence of hydroxyl groups and the ^{13}C NMR spectrum providing further verification of the structure.



SCHEME 2 Synthesis routes of compounds 9–12.

Similarly, when the silyloxypyrimidine derivative **8** was treated with β-D-glycopyranose pentaacetate in the presence of $SnCl_4$, it afforded the corresponding N-glycoside **11**. The ^{1}H NMR spectrum of **11** showed a doublet at 6.19 ppm ($J = 9.99$ Hz) assigned to the anomeric proton of the glucose moiety with a diaxial orientation of H-1′ and H-2′ indicating the β-configuration. The other protons of the glucopyranose ring resonate at 4.10–5.20 ppm,

while the four acetoxy groups appear as four singlets in the 1.92–2.10 ppm region providing further verification of the structure.[15]

Deprotection of **11** with methanolic ammonia at room temperature afforded the free N-glycoside **12** (Scheme 2). The ^{1}H NMR spectrum of the latter compound showed the anomeric proton as a doublet at δ 6.72 (J = 9.30 Hz) indicative for the β-D-configuration, and the signals of the other six carbon-bonded glucose protons appear as multiplets at δ 3.40–4.30, while the signals of the four hydroxyl groups of the glucose moiety are observed at δ 4.52–5.34 (exchangeable with D_2O). The ^{13}C NMR spectrum of **12** is characterized by the appearance of the C=O signal and the presence of a signal at δ 86.40 corresponding to the C-1′ atom. Another five signals at δ 61.63, 67.64, 69.89, 72.86, and 74.81 are assigned to C-6′, C-4′, C-2′, C-3′ and C-5′, respectively (see Experimental).

Moreover, the IR and ^{13}C NMR spectra of compounds **9–12** indicated that the site of attack was on the nitrogen since it behaves as a nucleophile which attacks an electrophilic carbon atom of an alkyl halide.

Antiviral Bioassays

Antiviral bioassays were carried out to test compounds **1–7** and **9–12**. The concentrations of the tested compounds, which exhibited 50% cytotoxicity (LD50) and the 50% effective antiviral concentration (EC50), were determined (Table 1) in addition to the cytotoxicity (CT) and the therapeutic index (TI).

Structural activity correlations of the obtained results indicated that substituted acyclic nucleoside analogs of pyrazolo[3,4-*d*]pyrimidine (derivatives **3–7**) revealed more anti-H5N1 activity than the parent pyrazole **1** and

TABLE 1 Antiviral activity against H5N1 virus of the prepared compounds by determination of both EC50 and LD50

Compound	LD50 (μg/ml)	EC50(μg/ml)/Avian influenza virus (H5N1)	Therapeutic index
1	80.5	102.5	0.785365854
2	87.08	209.69	0.415279699
3	133.9	179.87	0.74442653
4	145.11	193.6	0.749535124
5	101.39	126.17	0.80359832
6	100.33	103.93	0.965361301
7	79.54	122.7	0.648247759
9	125.85	148.32	0.848503236
10	175.34	188.6	0.929692471
11	163.07	204.31	0.79814987
12	192.31	201.12	0.956195306
Zanamivir	77	5	15.4

pyrazolo[3,4-*d*]pyrimidin-4-one derivative **2**. In general, the free cyclic N-nucleosides of pyrazolo[3,4-*d*]pyrimidine (derivatives **10** and **12**) showed the highest anti-H5N1 activity among the other tested compounds (Table 1).

EXPERIMENTAL

Chemistry

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-400 NMR spectrometer and chemical shifts were expressed as part per million; (δ values, ppm) against TMS as internal reference (Organic Chemistry, Chemnitz University of Technology, Chemnitz, Germany). 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).

Compound **1** was prepared according to a reported method.[19]

1-(9-Methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-d]pyrimidin-11-yl)-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one (2)

Compound **1** (0.358 g, 1 mmol) was heated under reflux in formic acid (30 ml, 85%) for 5 hours. The reaction mixture was cooled and poured into water. The formed solid was filtered off, dried, and recrystallized from dioxane to give compound **2** in 95% yield; m.p. 270–272°C; IR (KBr, ν, cm^{-1}): 3202 (NH) and 1677 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 2.37 (s, 3H, CH_3), 2.86–2.91 (m, 4H, $2CH_2$), 7.17–7.27 (m, 4H, 3Ar-H+ H3-pyrazole), 8.19 (s, 1H, H6-pyrimidine), 8.34 (d, 1H J = 7.60 Hz, 1H, Ar-H), 12.42 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, δ ppm): 20.59 (CH_3), 23.40 (CH_2), 29.28 (CH_2), 126.27–135.19 (13Ar-C), 147.10 (C-3), 154.84 (C-6), 158.17 (C-11′), 158.17 (C=O). MS, *m/z* (%): 386 (M^+, 84), 358 (M^+–CO, 16), 328 (14). Anal. calcd. for $C_{20}H_{14}N_6OS$ (386.09): C 62.16, H 3.65, N 21.75, S 8.30. Found: C 62.32, H 3.47, N 21.96, S 8.13.

General Procedure for the Synthesis of Compounds 3–7

To a solution of compound **2** (0.386 g, 1 mmol) in dry dimethylformamide (50 ml), 50% oil-immersed sodium hydride (0.20 g) was added. Thereafter the reaction mixture was stirred at room temperature for 1 hour, then ethyl iodide, 2-chloroethanol, 2-chloroethyl methyl ether, 2-chloroethyl

ethyl ether, or 2-chloroacetaldehyd dimethylacetal (1 mmol) was added, and the reaction mixtures were stirred at 70°C for 3, 5, 4, 3, and 2 hours, 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 **3**, **4**, **5**, **6** or **7**, respectively.

5-Ethyl-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d]pyrimidin-4-one (3)

Yield 76%; m.p. 215–217°C; IR (KBr, ν, cm^{-1}): 1691 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 1.40 (t, 3H, $J = 7.50$ Hz, NCH_2CH_3), 2.66 (s, 3H, CH_3), 2.84–2.95 (m, 4H, $2CH_2$), 3.29 (q, 2H, $J = 7.20$ Hz, NCH_2CH_3), 7.18–7.23 (m, 5H, 3Ar-H, H3-pyrazole, H6-pyrimidine), 8.01 (d, 1H, $J = 8.60$ Hz, Ar-H); ^{13}C NMR (DMSO-d_6, δ ppm): 14.26 (CH_3), 24.64 (CH_3), 25.35 (CH_2), 29.86 (CH_2), 34.12 (NCH_2), 125.21–138.40 (13Ar-C), 139.12 (C-3), 154.11 (C-6), 158.17 (C-11′), 163.01 (C=O). MS *m/z* (%): 414 (M^+, 64), 385 (61), 357 (14). Anal. calcd. for $C_{22}H_{18}N_6OS$ (414.13): C 63.75, H 4.38, N 20.28, S 7.74. Found: C 63.94, H 4.29, N 20.41, S 7.65.

5-(2-Hydroxyethyl)-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d]pyrimidin-4-one (4)

Yield 69%; oil; IR (KBr, ν, cm^{-1}): 3350–3300 (OH), 1672 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 2.74 (s, 3H, CH_3), 2.84–2.95 (m, 4H, $2CH_2$), 3.70–3.91 (m, 4H, $2CH_2$), 4.92 (bs, 1H, OH, D_2O exchangeable), 7.01–7.49 (m, 6H, 4Ar-H, H3-pyrazole, H6-pyrimidine); ^{13}C NMR (DMSO-d_6, δ ppm): 24.64 (CH_3), 25.35 (CH_2), 29.86 (CH_2), 60.32 (CH_2), 65.01 (CH_2), 120.29–148.86 (16Ar-C), 159.52 (C=O); Anal. calcd. for $C_{22}H_{18}N_6O_2S$ (430.49): C 61.38, H 4.21, N 19.52, S 7.45. Found: C 61.29, H 4.17, N 19.72, S 7.53.

5-(2-Methoxyethyl)-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d]pyrimidin-4-one (5)

Yield 72%; oil; IR (KBr, ν, cm^{-1}): 1690 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 2.63 (s, 3H, CH_3), 2.79–2.89 (m, 4H, $2CH_2$), 3.20 (s, 3H, OCH_3), 3.29 (t, 2H, $J = 5.20$ Hz, CH_2), 3.65 (t, 2H, $J = 7.50$ Hz, CH_2), 7.16–7.24 (m, 4H, 3Ar-H, H3-pyrazole), 7.92 (s, 1H, H6-pyrimidine), 8.24 (d, 1H, $J = 8$ Hz, Ar-H); ^{13}C NMR (DMSO-d_6, δ ppm): 22.60 (CH_3), 29.27 (CH_2), 31.83 (CH_2), 39.81 (OCH_3), 59.02 (CH_2), 70.33 (CH_2), 126.57–137.47 (13Ar-C), 140 (C-3), 146 (C-6), 149.92 (C-11′), 158.40 (C=O). MS *m/z* (%): 444 (M^+, 55), 413 (36), 390 (68), 362 (19). Anal. calcd. for $C_{23}H_{20}N_6O_2S$ (444.14): C 62.15, H 4.54, N 18.91, S 7.21. Found: C 62.29, H 4.67, N 18.72, S 7.13.

5-(2-Ethoxyethyl)-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno [2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d]pyrimidin-4-one (6)

Yield 57%; m.p. 160–162°C; IR (KBr, ν, cm^{-1}): 1685 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 1.27 (t, 3H, $J = 7.50$ Hz, OCH_2CH_3), 2.76 (s, 3H, CH_3), 2.89–3.00 (m, 4H, $2CH_2$), 3.39 (q, 2H, $J = 7.50$ Hz, OCH_2CH_3), 3.79 (t, 2H, $J = 7.50$ Hz, CH_2), 4.29 (t, 2H, $J = 7.50$ Hz, CH_2), 7.18–7.35 (m, 3H, 3Ar-H), 7.92 (s, 2H, Ar-H+H3-pyrazole), 8.20 (s, 1H, H6-pyrimidine); ^{13}C NMR (DMSO-d_6, δ ppm): 14.95 (CH_3), 23.49 (CH_3), 24.22 (CH_2), 30.05 (CH_2), 45.35 (OCH_2), 66.69 (CH_2), 68.22 (CH_2), 126.60–155.43 (16Ar-C), 158.22 (C=O). MS *m/z* (%): 458 (M^+, 49), 429 (18), 413 (21), 385 (59), 357 (15). Anal. calcd. for $C_{24}H_{22}N_6O_2S$ (458.15): C 62.86, H 4.84, N 18.33, S 6.99. Found: C 62.65, H 4.63, N 18.17, S 6.68.

5-(2,2-Dimethoxyethyl)-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno [2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d]pyrimidin-4-one (7)

Yield 68%; m.p. 213–215°C; IR (KBr, ν, cm^{-1}): 1680 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 2.77 (s, 3H, CH_3), 2.89–3.00 (m, 4H, $2CH_2$), 3.45–3.70 (m, 8H, $2OCH_3$+NCH_2), 4.86 (t, 1H, $J = 7.8$ Hz, CH), 7.16–7.31 (m, 4H, 3Ar-H+H3-pyrazole), 7.80 (s, 1H, H6-pyrimidine), 8.48 (d, 1H, $J = 7.50$ Hz, Ar-H); ^{13}C NMR (DMSO-d_6, δ ppm): 23.75 (CH_3), 24.29 (CH_2), 30.07 (CH_2), 40.0 ($2OCH_3$), 64.37 (CH_2), 100.89 (CH), 126.73–158.25 (16Ar-C), 162.86 (C=O). MS *m/z* (%): 474 (M^+, 68), 385 (72), 356 (12). Anal. calcd. for $C_{24}H_{22}N_6O_3S$ (474.15): C 60.75, H 4.67, N 17.71, S 6.76. Found: C 60.54, H 4.78, N 17.95, S 6.62.

5-(2′,3′,5′-Tri-O-benzoyl-β-D-ribofuranosyl)-1-(9-methyl-5,6-dihydronaphtho [1′,2′:4,5]thieno[2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d] pyrimidin-4-one (9)

Compound **2** (0.386 g, 1 mmol) was refluxed by stirring under anhydrous condition for 12 hours with hexamethyldisilazane (60 mL) and ammonium sulfate (0.02 g). The obtained clear solution was evaporated under reduced pressure and the resulting trimethylsilylated pyrimidine **8** was dissolved in dry 1,2-dichloromethane (40 mL) and a solution of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (1 mmol) and $SnCl_4$ (1.6 mL) in dry 1,2-dichloromethane (10 mL) was then added dropwise with stirring under inert atmosphere (nitrogen gas) until the reaction was judged complete by thin layer chromatography (TLC; 6 hours). The reaction mixture was poured into saturated $NaHCO_3$ solution and extracted with $CHCl_3$ (2×30 mL). The extracts were collected and dried over anhydrous Na_2SO_4, filtered and concentrated producing the crude nucleoside that was purified on silica gel column using *n*-hexane : ethyl acetate (4:1) as an eluent to give **9**. Yield 68%; m.p. 213–215°C; IR (KBr, ν, cm^{-1}): 1710, 1690 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 2.77 (s, 3H, CH_3), 2.65–2.80 (m, 4H, $2CH_2$), 4.10

(m, 1H, 4′-H), 4.48–4.88 (m, 4H, 5′-H_2, 2′-H, 3′-H), 6.69 (d, 1H, $J_{1',2'} = 4.40$ Hz, 1′-H), 7.21–7.60 (m, 18H, Ar-H), 7.81–8.10 (m, 3H, Ar-H, H3-pyrazole, H6-pyrimidine); ^{13}C NMR (DMSO-d_6, δ ppm): 22.67 (CH_3), 25.67 (CH_2), 30.27 (CH_2), 63.67 (C-5′), 70.69 (C-3′), 71.32 (C-2′), 79.93 (C-4′), 98.37 (C-l′), 124.75–129.83 (13Ar-C), 137 (C-3), 140 (C-6), 158.47, 165.02, 169.09, 169.57 (4C=O); Anal. calcd. for $C_{46}H_{34}N_6O_8S$ (830.88): C 66.50, H 4.12, N 10.11, S 3.86. Found: C 66.69, H 3.97, N 10.32, S 3.92.

5-(1-β-D-Ribofuranosyl)-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d]pyrimidin-4-one (10)

Dry ammonia gas was passed into a solution of protected nucleoside **9** (1 mmol) in dry methanol (20 mL) at 0°C for 0.5 hours, then the reaction mixture was stirred at room temperature until the reaction was judged complete by TLC (5 hours). The resulting mixture was then concentrated under reduced pressure at 40°C to afford a solid residue that was purified on silica gel column using chloroform : methanol (4:1) as an eluent to give **10**.

Yield 59%; oil; IR (KBr, ν, cm^{-1}): 3440–3230 (OH), 1665 (C=O); ^{1}H NMR (DMSO-d_6, δ ppm): 2.56 (s, 3H, CH_3), 2.65–2.80 (m, 4H, $2CH_2$), 4.23–4.40 (m, 3H, 4′-H, 5′-H_2), 4.50–4.62 (m, 2H, 2′-H, 3′-H), 4.82 (m, 1H, OH, D_2O exchangeable), 4.95 (m, 1H, OH, D_2O exchangeable), 5.15 (m, 1H, OH, D_2O exchangeable), 6.84 (d, 1H, $J_{1',2'} = 5.20$ Hz, 1′-H), 7.10–7.31 (m, 5H, 4Ar-H+ H3-pyrazole), 8.48 (s, 1H, H6-pyrimidine); ^{13}C NMR (DMSO-d_6, δ ppm): 25.32 (CH_3), 25.67 (CH_2), 29.62 (CH_2), 64.22 (C-5′), 72.86 (C-3′), 75.24 (C-2′), 81.35 (C-4′), 92.05 (C-l′), 115.52–150.15 (16Ar-C), 158.47 (C=O); Anal. calcd. for $C_{25}H_{22}N_6O_5S$ (518.56): C 57.91, H 4.28, N 16.21, S 6.18. Found: C 58.19, H 4.17, N 16.32, S 6.16.

5-(2′,3′,4′,6′-Tetra-O-acetyl-β-D-glucopyranosyl)-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d]pyrimidin-4-one (11)

The same procedure as in preparation of compound **9** was performed, but 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (1 mmol) was used to give product **11**.

Yield 66%; m.p. 195–197°C; IR (KBr, ν, cm^{-1}): 1715, 1680 (C=O). ^{1}H NMR (DMSO-d_6, δ ppm): 1.92, 1.98, 2.01, 2.10 (4s, 12H, $4CH_3CO$), 2.60 (s, 3H, CH_3), 2.95–3.00 (m, 4H, $2CH_2$), 4.10–4.20 (m, 2H, 6′-H_2), 4.85–5.20 (m, 4H, 5′-H, 4′-H, 3′-H, 2′-H), 6.19 (d, 1H, $J_{1',2'} = 9.99$ Hz, 1′-H), 7.20–7.31 (m, 4H, 3Ar-H+H3-pyrazole), 8.29–8.40 (m, 2H, Ar-H+H6-pyrimidine); ^{13}C NMR (DMSO-d_6, δ ppm): 20.60–20.73 ($4CH_3$), 24.32 (CH_3), 29.67 (CH_2), 31.62 (CH_2), 62.68 (C-6′), 68.19 (C-4′), 69.49 (C-2′), 74.13 (C-3′), 74.81 (C-5′), 89.98 (C-1′), 128.17–159.50 (16Ar-C), 164.15, 169.34, 169.54, 169.93, 170.54 (5C=O); Anal. calcd. for $C_{34}H_{32}N_6O_{10}S$ (716.73): C 56.98, H 4.50, N 11.73, S 4.47. Found: C 57.12, H 4.57, N 11.62, S 4.56.

5-(β -D-Glucopyranosyl)-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno [2,3-d]pyrimidin-11-yl)-1,5-dihydropyrazolo[3,4-d]pyrimidin-4-one (12)

The same procedure as in preparation of compound **10** was used to give product **12** from **11**.

Yield 74%; oil; IR (KBr, ν, cm^{-1}): 3500–3200 (OH), 1685 (CO); ^1H NMR (DMSO-d_6, δ ppm): 2.77 (s, 3H, CH_3), 2.95–3.00 (m, 4H, $2CH_2$), 3.40–3.60 (m, 3H, 6′-H_2, H-5′), 4.10–4.30 (m, 3H, H-4′, H-3′, H-2′), 4.52 (m, 1H, OH, D_2O exchangeable), 5.03 (m, 1H, OH, D_2O exchangeable), 5.17 (m, 1H, OH, D_2O exchangeable), 5.34 (m, 1H, OH, D_2O exchangeable), 6.72 (d, 1H, $J_{1',2'} = 9.30$ Hz, H-1′), 6.91–7.31 (m, 5H, 4Ar-H+H3-pyrazole), 8.48 (s, 1H, H6-pyrimidine); ^{13}C NMR (DMSO-d_6, δ ppm): 22.68 (CH_3), 25.60 (CH_2), 29.66 (CH_2), 61.63 (C-6′), 67.64 (C-4′), 69.89 (C-2′), 72.86 (C-3′), 74.81 (C-5′), 86.40 (C-1′), 128.17–149.50 (13Ar-C), 164.15 (C=O); Anal. calcd. for $C_{26}H_{24}N_6O_6S$ (548.58): C 56.93, H 4.41, N 15.32, S 5.84. Found: C 56.99, H 4.37, N 15.45, S 4.86.

ANTIVIRAL BIOASSAY

MTT Assay (Cytotoxicity Assay)

Samples were diluted with Dulbecco's Modified Eagle's Medium (DMEM) to desired concentrations (10, 20, 30, and 40 $\mu g/\mu l$). Stock solutions of the test compounds were prepared in 10% DMSO in dd H_2O. The cytotoxic activity of the extracts were tested in Madin Darby Canine kidney (MDCK) cells by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method[22] 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 incubated for 24 hours at 37°C in 5%CO_2. After 24 hours, cells were treated with various concentrations of the tested compounds in triplicates. After an additional 24 hours, the supernatant was discarded and cell monolayers were washed with sterile phosphate buffer saline (PBS) three times and MTT solution (20 μl of 5 mg/ml stock solution) was added to each well and incubated at 37°C for 4 hours followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 μl of acidified isopropanol (0.04 M HCl in absolute isopropanol). Absorbance of formazan solutions were measured at λ_{max} 540 nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation.

$$\%\text{Cytotoxicity} = \frac{\text{Absorbance of cells without treatment} - \text{Absorbance of cells with treatment}}{\text{Absorbance of cells without treatment}}$$

The plot of the percentage of cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (LD50).

EC50 and Therapeutic Index

The antiviral activity of the tested compounds was determined using cytopathogenicity (CPE) assay against low pathogenic reassotant avian influenza virus (rH5N1). Stock solutions of the test compounds were prepared in DMSO at a concentration of 10 mg/ml. Cells grown to confluency in 96-well plates were infected with 100 μl of stock virus. After an adsorption period of 2 hours at 37°C, virus was removed and serial dilutions of the tested compounds were added, then maintenance DMEM with 2% FBS was added (100 μl/well). The cultures were further incubated at 37°C for 3 days, until complete CPE was observed in the infected and untreated virus control. The determination of the anti-influenza virus activity of the tested compounds was based on virus-induced cytopathogenicity of H5N1-infected MDCK cells, measured at day 3 post virus infection by the MTT colorimetric method.[23] An absorbance of formazan was detected by a multi-well plate reader at 540 nm with 620 nm reference wavelength. The results were expressed as the 50% effective concentration (EC50). The 50% effective antiviral concentration (EC50) was defined as the compound concentration required for protecting 50% of the virus-infected cells against viral cytopathogenicity. The therapeutic index was calculated by dividing LD50 on EC50.

Plaque Reduction Assay

In a six-well plate, confluent MDCK cells were infected with a preincubated mixture of 100 μl of avian influenza H5N1 virus (80–100 plaques/well), 100 μl of DMEM [containing 2% antibiotics and 1 μg/ml of *L*-1-tosylamido-2-phenylethyl chloromethyl ketone [TCPK] and different concentration of each compound (10, 20, 30, and 40 μg/μl). The plates were incubated for 45 minutes at 37°C in 5%CO_2 to allow virus adsorption. After adsorption, 2 ml of agarose overlayer in 2X DMEM containing 1% FBS was added to each well and mixed. The cultures were incubated at 37°C in 5%CO_2 for 3 to 4 days. Plaques were fixed with 3.7% formalin in phosphate-buffered saline for 2 hours followed by removal of the agarose over layer and staining with 0.1% crystal violet in distilled water. Plaques were counted manually from duplicate wells based on plaque number but not plaque size. Viral counts and percentage of virus reduction were calculated according to Hayden et al.[24]

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