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Synthesis and antiviral evaluation of some new pyrazole and fused pyrazolopyrimidine derivatives

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ABSTRACT

Some novel substituted pyrazole and pyrazolo[3,4-*d*]pyrimidine derivatives **2**, **4**, **8**, and **9** were synthesized. Also, some acyclic *S*-nucleosides of pyrazolo[3,4-*d*]pyrimidine derivatives **10**–**13** were prepared via reaction of pyrazolo[3,4-*d*]pyrimidine-4(3*H*)-thione derivative **9** with some acyclic sugars. Moreover, the *N*-nucleoside derivative **14** was prepared via reaction of compound **8** with glucosamine hydrochloride. The antiviral evaluation of some selected new products showed that they have promising antiviral activity against hepatitis-A virus (HAV) and herpes simplex virus type-1 (HSV-1).

1. Introduction

The pyrazole ring has attracted great attention[1] as it has become fairly accessible and it shows diverse properties. These heterocyclic compounds are not only interesting from a viewpoint of biological activities as potential inhibitors of HIV,[2] herbicides,[3] bactericides,[4] and analgesic drugs,[5] but also important and useful as starting materials for the synthesis of other fused heterocyclic pyrazolo[3,4-*d*]pyrimidine derivatives of considerable chemical and pharmacological importance such as purine analogs.[6,7] Several substituted pyrazolo[3,4-*d*]pyrimidine derivatives have xanthine oxidase inhibitor activity[7,8] like allopurinol which was first synthesized by Robins in 1956,[9] and is still the drug for the treatment of hyperuricemia and gouty arthritic disease.[7–10] Also, it was reported[11] that some pyrazolo[3,4-*d*]pyrimidine derivatives demonstrated significant antimicrobial,[12] antimycobacterial activity,[13] antitumor,[14] and antiviral activity.[15] On the other hand, few cyclic pyrazolo[3,4-*d*]pyrimidine nucleosides were reported in the literature,[16] while other acyclic fused pyrimidine nucleosides were reported to be potent anti-herpetic drugs with selective activity against herpes simplex virus (HSV-1 and HSV-2)[17,18] and varicella zoster virus (VZV).[18] However, there is still a great need and interest to prepare more active cyclic and acyclic pyrimidine nucleosides with fewer side effects than those observed for the already known derivatives.

The biological activity and structure–activity relationship (SAR) of some newly synthesized compounds were evaluated in comparison to those of amantadine and acyclovir, and these compounds were found to possess potent antiviral activities (see Figs. 1 and 2).

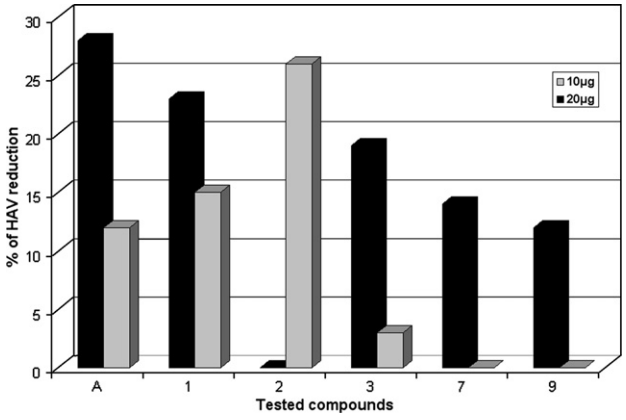

Figure 1. Effect of some novel pyrazole and fused pyrazolopyrimidine derivatives on HAV (MBB cell culture strain) in comparison with amantadine (A#) as a control.

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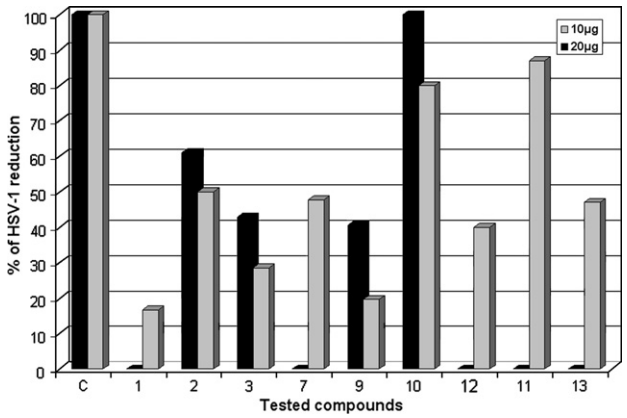


Figure 2. Effect of some novel pyrazole and fused pyrazolopyrimidine derivatives on HSV-1 (MBB cell culture strain) in comparison with acyclovir (C*) as a control.

2. Discussion

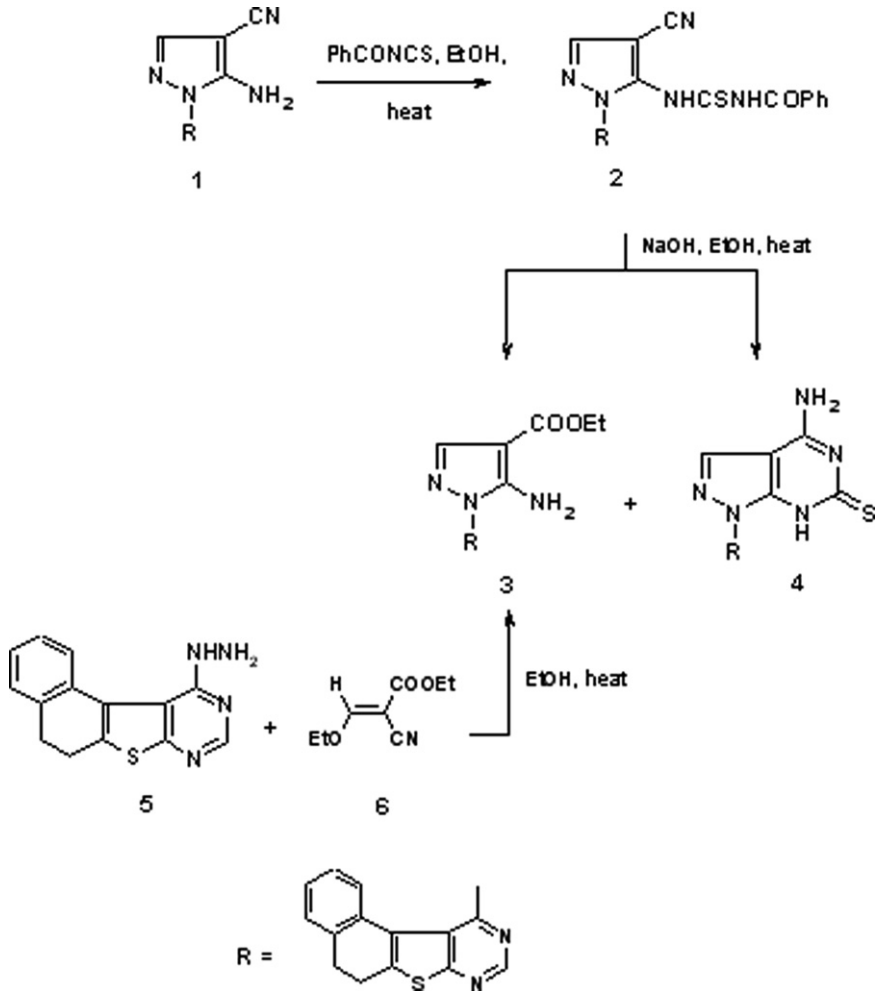
2.1. Chemistry

The reaction of 5-amino-1-(5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazole-4-carbonitrile (**1**)[12] with benzoyl isothiocyanate (generated in situ) gave the corresponding benzoylthiourea derivative **2**. The structure of compound **2** was confirmed by the elemental analysis and spectral data (see Section 4). Alkaline hydrolysis of the latter compound with sodium hydroxide did not afford only product assigned to the structure of compound **3**, but also afforded the cyclized 4-aminopyrazolo[3,4-*d*]pyrimidine-6(5*H*)-thione derivative **4**. Moreover, compound **3** could be obtained from the reaction of **5** with ethyl-(ethoxymethylene)cyanoacetate **6**, which is identical in all respects (physical and spectral data) with that obtained previously (Scheme 1).

On the other hand, compound **7**[19] was converted to its corresponding 4-chloro derivative **8**, when heated with phosphorus oxychloride under reflux. The mass spectrum of compound **8** gave fragments showing the isotopic pattern due to the presence of chlorine atom (see Section 4).

Also, on heating compound **7**[19] with phosphorus pentasulfide[17] or compound **8** with thiourea in ethanol,[20] the reaction afforded 1-(5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-4(3*H*)-thione (**9**). Analytical and spectral data are in agreement with the proposed structure of compound **9**, particularly the absence of C=O and the presence of C=S signals in the IR and ^{13}C NMR spectra (see Section 4).

Since the synthesis of acyclovir, as one of the most potent antiherpetic drug by Schaffer et al. in 1978,[21] many attempts have been directed by nucleoside chemists to prepare a lot of relative compounds with various side chains and glycons[17,21–23]; however,



Scheme 1.

acyclonucleosides of pyrazolo[3,4-*d*]pyrimidine derivatives were rarely reported in the literature.[24] Thus, in continuation of our previous work[17,22,24–26] in preparing various cyclic and acyclic nucleosides of different heterocyclic compounds, we describe in this report the synthesis of some *S*- and *N*-nucleosides related to pyrazolo[3,4-*d*]pyrimidine derivatives. So, when the potassium salt (generated in situ) of compound **9** (which then exists in the thiol tautomeric form under the condition of the reaction) was treated with 2-chloroethyl methyl ether, chloroacetaldehyde dimethylacetal, 2-chloroehanol, and 2-(2-chloroethoxy)ethanol afforded the corresponding *S*-acyclic nucleosides **10**, **11**, **12**, and **13**, respectively. The absence of C=S signals in ^{13}C NMR spectra of compounds **10** and **11** confirmed that the site of the attack was on the *S*- and not on the *N*- atom. Moreover, the presence of methoxyethyl and dimethoxyethyl protons and the absence of the NH signal in ^{1}H NMR spectra of compounds **10** and **11** confirmed their structures. Similarly, the IR spectra of compounds **12** and **13** revealed signals indicative for the OH groups, while their ^{1}H NMR spectra revealed signals indicative for the OH and CH_2 groups (see Section 4).

On the other hand, the reactivity of compound **8** toward *N*-nucleophiles was investigated. So, when compound **8** was refluxed with glucosamine hydrochloride in the presence of triethylamine, the reaction afforded *N*-[1-(5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-glucosamine (**14**). The proposed structure of compound **14** was elucidated on the basis of analytical and spectral data (see Section 4, Scheme 2).

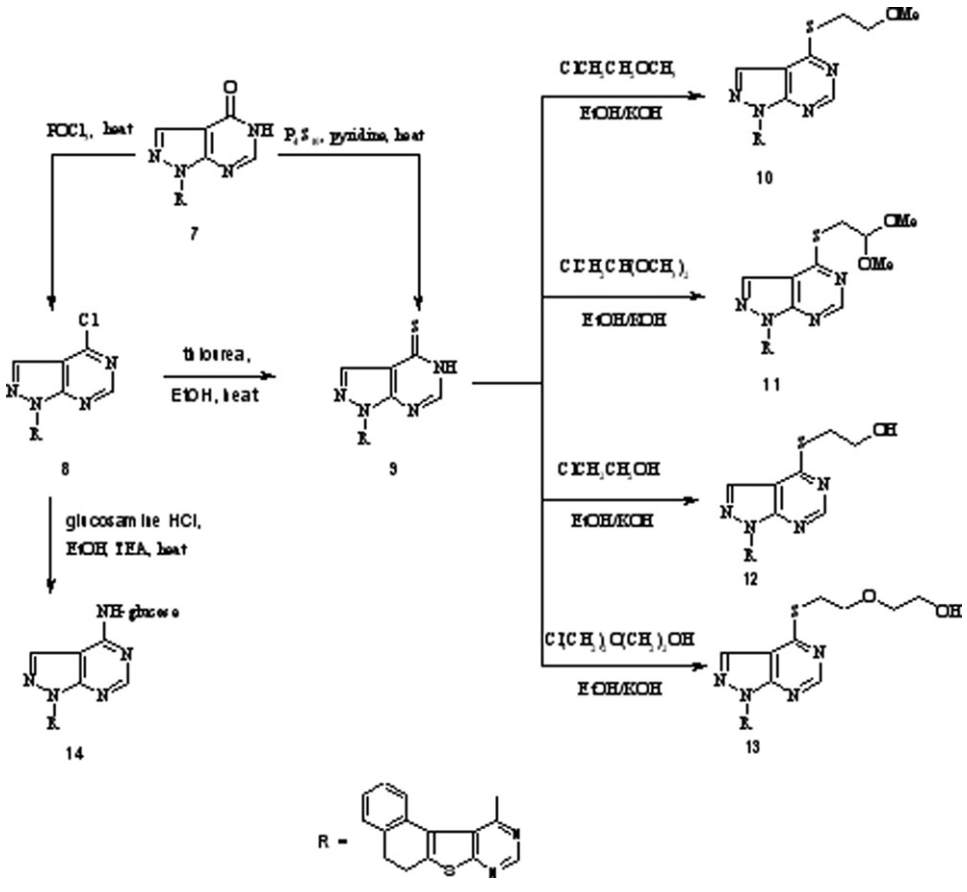
2.2. Antiviral bioassay

Plaque infectivity assay was carried out to test compounds **1**, **2**, **3**, **7**, **9** and **10–13** for antiviral activity. The test was performed to include the three possibilities for antiviral activity; virucidal effect, virus adsorption and effect on virus replication for both HAV and HSV-1 at concentrations 10 μg/10^5 and 20 μg/10^5 cells. It was obvious that at a concentration of 10 μg/10^5 cells, enaminonitrile and the thiourethane of the pyrazole derivative (compounds **1** and **2**) revealed the highest anti-HAV activity in comparison with amantadine (A$^{\#}$) as a control. While at a concentration of 20 μg/10^5 cells, compounds **1** and **3** revealed the highest anti-HAV activity in comparison with the other tested compounds. On the other hand, at a concentration of 10 μg/10^5 cells, the thiourethane and *S*-acyclic nucleosides (compounds **2**, **10**, and **11**) revealed the highest anti-HSV-1 activity in comparison with the other tested compounds. While at a concentration of 20 μg/10^5 cells, compounds **2** and **10** revealed the highest anti-HSV-1 activity in comparison with the other tested compounds.

3. Conclusions

Structural activities correlation of the obtained results revealed that at both concentrations the substituted pyrazole derivatives (compounds **1**–**3**) revealed higher anti-HAV activity than the fused pyrazolopyrimidine derivatives (**7** and **9**), that is, fusion of pyrimidine to the pyrazole ring decreases the anti-HAV activity.

Moreover, at a concentration of 10 μg/10^5 cells, addition of fused pyrimidine to the pyrazole ring (compounds **7** and **9**) slightly increases the anti-HSV-1 activity. On the other hand, at concentration of 20 μg/10^5 cells, the substituted pyrazole and fused pyrazolopyrimidine derivatives revealed higher anti-HSV-1 activity than *S*-acyclic nucleoside derivatives. (compounds **11**–**13**). In general, at higher concentrations all tested compounds revealed lower antiviral activity than the controls (amantadine and acyclovir).



Scheme 2.

4. Experimental

4.1. Chemistry

4.1.1. General

All melting points are uncorrected and measured using Electrothermal IA 9100 apparatus (Shimadzu, Japan). IR spectra were recorded as potassium bromide pellets on a Perkin-Elmer 1650 Spectrophotometer, National Research Centre, Cairo, Egypt. ^{1}H NMR and ^{13}C NMR spectra were determined on a Jeol-Ex-300 NMR spectrometer, and chemical shifts were expressed as part per million; ppm (δ values) against TMS as internal reference (Faculty of Science, Cairo University, Cairo, Egypt). Mass spectra were recorded on EI + Q1 MSLMR UPLR, National Research Centre, Cairo, Egypt. Microanalyses were operated using Mario Elmentar apparatus, Organic Microanalysis Unit, National Research Centre, Cairo, Egypt, and the results were within the accepted range of the calculated values. Column Chromatography was performed on (Merck) Silica gel 60 (particle size 0.06–0.20 mm).

Compounds **1**, **3**, **5**, **6**,[12] and **7**[19] were prepared as reported in the literature.

4.1.2. *N*-Benzoyl-*N*′-[4-cyano-1-(5,6-dihydronaphtho[1′,2′:4,5]-thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazol-5-yl]thiourea (2)

A mixture of benzoyl chloride (0.01 mol), and ammonium isothiocyanate (0.01 mol) was heated at reflux temperature in dry acetone (20 ml) for 15 min, then compound **1** was added and the reaction mixture was heated at reflux temperature for 2 h, poured into ice water and the deposited solid was filtered off, washed several times with water, dried and recrystallized from dioxane to give compound **2**. Yield 59%, mp 240–242 °C; IR (KBr) ν_{max} 3286, 3133 (2NH), 2204 (C≡N), 1629 (C=O) and 1330 (C=S) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 2.9–3.1 ppm (m, 4H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2), 6.0 (s, 1H, NH, D_2O exchangeable), 6.4–7.4 (m, 9H, Ar–H), 7.6 (s, 1H, C_3–H), 7.9 (s, 1H, NH, D_2O exchangeable), 9.1 (s, 1H, $C_{9'}$–H). Anal. Calcd for $C_{26}H_{17}N_7OS_2$ (507.59): C, 61.52; H, 3.38; N, 19.32; S, 12.63. Found: C, 61.72, H, 3.63, N, 19.18, S, 12.24.

4.1.3. General procedure for the synthesis of compounds 3 and 4

Compound **2** (0.01 mol) was heated at reflux temperature in 20 ml of alcoholic sodium hydroxide solution (sodium hydroxide 1.68 g, 7 ml water) for 4 h, then evaporated under reduced pressure, and the residue was purified on silica gel column using petroleum ether 40–60 °C/ethyl acetate (7:3) as an eluent to give products **3** and **4**. Compound **3** obtained here is identical in all respects with that obtained in previous report.[12]

4.1.3.1. 4-Amino-1-(5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-*d*]-pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin–6(5H)-thione (4).

Yield 55%, mp 298–301 °C; IR ν_{max} 3400, 3200, and 3071 (NH_2/NH) cm^{-1} and disappearance of the C≡N group; ^{1}H NMR (DMSO-d_6): δ 2.8–2.9 ppm (m, 4H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2), 7.1–7.3 (m, 6H, C_3–H, 3Ar–H, NH_2, D_2O exchangeable), 8.1 (s, 1H, $C_{9'}$–H), 8.35 (d, 1H, Ar–H, J = 7.8 Hz), 12.4 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 24.4 (C-5′), 29.3 (C-6′), 129–156 (sp^2 carbon atoms), 167.3 (C=S). Anal. Calcd for $C_{19}H_{13}N_7S_2$ (403.49): C, 56.56; H, 3.25; N, 24.30; S, 15.89. Found: C, 56.32; H, 3.61; N, 24.76; S, 15.36.

4.1.4. 4-Chloro-1-(5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-*d*] pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (8)

Compound **7**[19] (0.01 mol) was heated at reflux in phosphorus oxychloride (20 ml) for 2 h. The solution was cooled and poured with stirring onto ice and the solid that formed was filtered off, washed several times with water, dried and purified on silica gel column using petroleum ether/ethyl acetate (3:1) as an eluent to give compound **8**. Yield 55%, mp 190–192 °C; IR ν_{max} 1526 cm^{-1} of the C=N, and absence of the C=O and NH groups; ^{1}H NMR (DMSO-d_6): δ 2.9–3.0 ppm (m, 4H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2), 7.2–7.3 (m, 4H, 3Ar–H + C_3–H), 8.0 (s, 1H, $C_{9'}$–H), 8.35 (d, 1H, Ar–H, J = 7.5 Hz), 8.9 (s, 1H, C_6–H); EIMS, m/z (%): 392 (M^+, ^{37}Cl, 39.98), 390 (M^+, ^{35}Cl, 100). Anal. Calcd for $C_{19}H_{11}ClN_6S$ (390.05): C, 58.39; H, 2.84; N, 21.50; S, 8.20. Found: C, 58.51; H, 2.61; N, 21.38; S, 8.41.

4.1.5. 1-(5,6-Dihydronaphtho[1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-4(3*H*)-thione (9)

(i) Compound **7**[19] (0.01 mol) was heated at reflux temperature in (20 ml) dry pyridine containing phosphorus pentasulfide (0.01 mol) for 5 h. The solution was cooled and poured with stirring onto ice, acidified with dil hydrochloric acid and the solid that formed was filtered off, washed several times with water, dried and recrystallized from dimethylformamide to give compound **9** in 75% yield.

(ii) Compound **8** (0.01 mol) and thiourea (0.01 mol) were heated at reflux temperature in dry ethanol (40 ml) for 4 h. The solid product was collected and recrystallized from dimethylformamide to give compound **9** in 70% yield, mp 245–247 °C; IR ν_{max} 3127 (NH) and 1221 cm^{-1} of the C=S group; ^{1}H NMR (DMSO-d_6): δ 2.8–2.9 ppm (m, 4H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2), 7.1–7.3 (m, 5H, 3Ar–H + $C_{9'}$–H + C_3–H), 8.0 (d, 1H, Ar–H, J = 9.0 Hz), 8.2 (s, 1H, C_6–H), 13.8 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): δ 24.6 (C-5′), 29 (C-6′), 127–157 (sp^2 carbon atoms), 164 (C=S). Anal. Calcd for $C_{19}H_{12}N_6S_2$ (388.47): C, 58.74; H, 3.11; N, 21.63; S, 16.51. Found: C, 58.31; H, 3.51; N, 21.77; S, 16.21.

4.1.6. General procedure for the synthesis of 10, 11, 12, and 13

To a solution of dry ethanol (20 ml) containing KOH (0.01 mol), compound **9** (0.01 mol) was added, then the reaction mixture was stirred at room temperature for 15 min, then 2-chloroethyl methyl ether, chloroacetaldehyde dimethylacetal, 2-chloroehanol, or 2-(2-chloroethoxy)-ethanol (0.01 mol) was added and the reaction mixtures were stirred at 70 °C for 3, 4, 3, and 2 h, respectively. The reaction mixtures were evaporated under reduced pressure and the residues were purified on silica gel column to give compounds **10**, **11**, **12**, and **13**, respectively.

4.1.6.1. 4-(2-Methoxyethylsulfanyl)-1-(5,6-dihydronaphtho [1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*] pyrimidine (10).

Yield 65%, mp 217–219 °C; IR ν_{max} absence of the NH and C=S signals; ^{1}H NMR (DMSO-d_6): δ 2.9–3.0 ppm (m, 4H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2), 3.5 (s, 3H, OCH_3), 4.5–4.6 (m, 4H, $2CH_2$), 7.1–7.6 (m, 5H, 4Ar–H + C_3–H), 7.9 (s, 1H, $C_{9'}$–H), 8.5 (s, 1H, C_6–H); ^{13}C NMR (DMSO-d_6) δ 24.6 (C-5′), 29 (C-6′), 29.78 (OCH_3), 58.89 (CH_2), 71.21 (CH_2), 127–157 (sp^2 carbon atoms), 164 (C=S). Anal. Calcd for $C_{22}H_{18}N_6OS_2$ (446.55): C, 59.17; H, 4.06; N, 18.82; S, 14.36. Found: C, 59.52; H, 4.41; N, 18.63; S, 14.06.

4.1.6.2. 4-(Dimethoxyethylsulfanyl)-1-(5,6-dihydronaphtho [1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*] pyrimidine (11).

Yield 33%, oil; IR ν_{max} 1591 cm^{-1} of the C=N group, and absence of the NH and C=S signals; ^{1}H NMR (DMSO-d_6): δ 2.05–2.1 ppm (m, 2H, CH_2), 2.6 (t, 1H, CH), 2.9–3.0 (m, 4H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2), 3.45 (s, 6H, $2OCH_3$), 7.2–7.8 (m, 6H, 4Ar–H + C_3–H + $C_{9'}$–H), 9.2 (s, 1H, C_6–H); ^{13}C NMR (DMSO-d_6): δ 19 (C-5′), 23.5 (C-6′), 30.0 (CH_2), 40.0 ($2OCH_3$), 58 (CH), 127–145 (sp^2 carbon atoms). Anal. Calcd for $C_{23}H_{20}N_6O_2S_2$ (476.58): C, 57.96; H, 4.23; N, 17.63; S, 13.46. Found: C, 57.72; H, 4.52; N, 17.36; S, 13.77.

4.1.6.3. 4-(2-Hydroxyethylsulfanyl)-1-(5,6-dihydronaphtho [1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*] pyrimidine (12).

Yield 53%, oil; IR ν_{max} 3430–3300 cm^{-1} of OH,

1591 cm^{-1} of the C=N group, and absence of the NH and C=S signals; ^1H NMR (DMSO-d_6): δ 2.5–2.6 ppm (m, 4 H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2), 2.91–2.95 (m, 2H, CH_2), 3.6–3.8 (m, 2H, CH_2), 5.0 (br s, OH, D_2O exchangeable), 7.3–7.8 (m, 6H, 4Ar–H + C_3–H + $C_{9'}$–H), 9.2 (s, 1H, C_6–H). Anal. Calcd for $C_{21}H_{16}N_6OS_2$ (432.52): C, 58.31; H, 3.73; N, 19.43; S, 14.83. Found: C, 58.75; H, 3.42; N, 19.12; S, 14.96.

4.1.6.4. 4-(Hydroxyethoxyethylsulfanyl)-1-(5,6-dihydro-naphtho[1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (13). Yield 47%, oil; IR ν_{max} 3430–3300 cm^{-1} of broad OH, 1591 cm^{-1} of C=N, and the absence of the NH and C=S signals; ^1H NMR (DMSO-d_6): δ 2.1 ppm (t, 2H, CH_2), 2.5–2.6 ppm (m, 4H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2), 2.91–2.95 (m, 2H, CH_2), 3.6–3.8 (m, 2H, CH_2), 5.0 (s, OH, D_2O exchangeable), 7.3–7.8 (m, 6H, 4Ar–H + C_3–H + $C_{9'}$–H), 9.2 (s, 1H, C_6–H). Anal. Calcd for $C_{23}H_{20}N_6O_2S_2$ (476.58): C, 57.96; H, 4.23; N, 17.63; S, 13.46. Found: C, 57.62; H, 4.65; N, 17.98; S, 13.03.

4.1.7. *N*-[1-(5,6-Dihydronaphtho[1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin]glucosamine (14)

A solution of compound **8** (0.01 mol) in dry ethanol (20 ml) was treated with glucosamine hydrochloride (0.01 mol) in the presence of triethylamine (2 drops) as a catalytic amount. The reaction mixture was refluxed for 3 h, and the solid product was collected by filtration on hot, dried, and recrystallized from dioxane to give compound **14**. Yield 45%, mp 224–226 °C; IR ν_{max} 3250–3400 (NH + OH), 1745 cm^{-1} (C=O); ^1H NMR (DMSO-d_6): δ 2.9–3.3 ppm (m, 8H, $C_{5'}$–CH_2 + $C_{6'}$–CH_2 + 4CH), 3.4–3.45 (m, 2 H, CH_2O), 3.8 (s, 1H, OH, D_2O exchangeable), 4.3–4.6 (m, 2H, 2OH, D_2O exchangeable), 4.9 (s, 1H, OH, D_2O exchangeable), 5.9–5.95 (m, H, COCH), 6.0 (s, 1H, OH, D_2O exchangeable), 6.6 (s, 1H, NH, D_2O exchangeable), 7.1–7.6 (m, 5H, 4Ar–H + C_3–H), 8.4 (s, 1H, C_6–H), 8.9 (s, 1H, $C_{9'}$–H). Anal. Calcd for $C_{24}H_{23}N_7O_5S$ (521.55): C, 55.27; H, 4.44; N, 18.80; S, 15.34. Found: C, 55.39; H, 4.53; N, 18.67; S, 15.71.

4.2. Antiviral bioassay

4.2.1. Preparation of synthetic compounds for bioassay

Hundred milligrams of each tested compound were dissolved in 1 ml of 10% DMSO in water. The final concentration was 100 μg/μl (Stock solution). The dissolved stock solutions were decontaminated by addition of 50 μg/ml antibiotic–antimycotic mixture (10,000 U penicillin G sodium, 10,000 μg streptomycin sulfates and 250 μg amphotericin B, PAA Laboratories GmbH, Austria).

4.2.2. Cell culture

African green monkey kidney-derived cells (Vero) and human hepatoma cell line (HepG2) were used. Cells were propagated in Dulbeccos' minimal essential medium (DMEM) supplemented with 10% foetal bovine serum, 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 0.2 μm pore size nitrocellulose membrane.

4.2.3. Viruses

Herpes simplex virus type-1 (HSV-1) and hepatitis-A virus (HAV, MBB-cell culture adapted strain) were obtained from Environmental Virology Lab., Department of Water Pollution Research, National Research Centre, Cairo, Egypt.

4.2.4. Cytotoxicity assay

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

4.2.5. Plaque reduction infectivity assay

A 6-well plate was cultivated with cell culture (10^5 cell/ml) and incubated for 2 days at 37 °C. HSV-1 and HAV were diluted to give 10^4 PFU/ml final concentrations for each virus and mixed with the tested compound at 10 μg/10^5 and 20 μg/10^5 cells, and incubated overnight at 4 °C. Growth medium was removed from the multiwell plate and 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% agarose was overlaid the cell sheets. The plates were left to solidify and incubated at 37 °C until the development of virus plaques. Cell sheets were fixed in 10% formaline solution for 2 h and stained with crystal violet stain. Control virus and cells were treated identically without chemical compound. Virus plaques were counted, and the percentage of reduction was calculated.[27]

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