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Synthesis and anticancer evaluation of some fused pyrazolopyrimidines and their *S*-acyclic nucleosides

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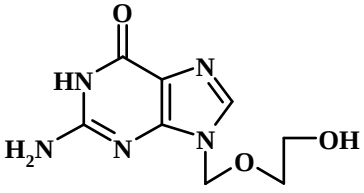
ABSTRACT

6-Mercapto-1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-one ***(2)*** *was prepared and used as a key compound for the preparation of some S-acyclic nucleosides of pyrazolo[3,4-d]pyrimidines* ***4-9****. Also, the synthesis and structure characterization of pyrazolo[3,4-d][1,3]thiazolidino[3,2-a]pyrimidine* ***10*** *and pyrazolo[3,4-d][1,3]-thiazolo[3,2-a]pyrimidine* ***11*** *were described. Moreover, the cytotoxicity and in vitro anticancer evaluation of the prepared compounds have also been assessed against breast MCF-7 cancer and liver HepG2 cancer cell lines with investigation the effect of the synthesized compounds on the expression of urokinase plasminogen activator (uPA). The results revealed that, compounds* ***8*** *and* ***7*** *revealed promising anticancer activity compared to the activity of the commonly used anticancer drug, doxorubicin with inhibiting the expression of uPA.*

Keywords: Pyrazoles; Pyrazolopyrimidines; pyrazolothiazolidinopyrimidine; pyrazolothiazolopyrimidine; Acyclic *S*-nucleosides, *in vitro* anticancer evaluation.

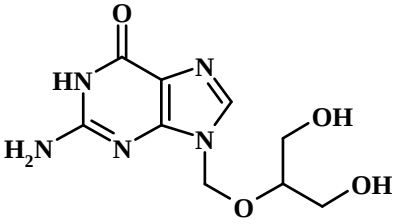
INTRODUCTION

Acyclic nucleosides are a group of nucleosides, which differ only from the parent ribonucleosides by the absence of ring structure of the pentosyl residue. The general feature of the important members of this class of nucleosides is the absence of one or more of the bonds of the pentose moiety to give an open chain residue, i.e., they possess portion of the pentose residue [1-4].



I

Acyclovir



II

Ganciclovir

Many of these analogues have been synthesized and co mprehensively covered in the literature as they include the most important group of antiviral agents used now like acyclovir I and ganciclovir II which were reported to be a potent antiherpetic drug with selective activity against Herpes Simplex Viruses, Varicella Zoster Virus (VZV), Human Cytomegalo Virus (HCMV), and Epstein-Barr Virus (EBV) [5-7]. Also, some recent reports revealed that acyclic nucleosides have antimicrobial [8], antiviral [9], and anticancer agents [10]. Nucleosides were reported to be of great value from the biological point of view and many of their derivatives have been synthesized recently as potential antimicrobialial agents [11] and tested as a novel type of histamine H-3 antagonist [12] and were tested also against HSV-1 and HSV-2 [13].

In addition, pyrazolopyrimidines are the fused heterocyclic ring systems which structurally resemble purines which prompted biological investigations to assess their potential therapeutic significance and they are known to play a crucial role in numerous disease conditions [14-17]. Moreover, recent publications have emerged describing that these molecules are known to exhibit pharmacogical activities and hence several pyrazolo[3,4-*d*]pyrimidine derivatives revealed promising activity as tuberculostatic [18], antiviral [19], antimicrobial [20], anti-inflammatory [21] and divulged anticancer potential [22,23].

Based on the above mentioned research results, the goal of this study is to synthesize some novel pyrazolopyrimidines, fused pyrazolopyrimidines and their acyclic *S*-nucleosides to obtain new compounds which are expected to possess notable pharmacological applications.

MATERIALS AND METHODS

3. Experimental

3.1. 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 (Perkin-Elmer, Norwalk, CT, USA). ^{1}H NMR and ^{13}C NMR spectra were determined on a Jeol-Ex-400 NMR spectrometer (JEOL, Tokyo, Japan) and chemical shifts were expressed as part per million; (δ values, ppm) against TMS as internal reference. Mass spectra were recorded on VG 2AM-3F mass spectrometer (Thermo Electron Corporation, USA). Microanalyses were operated using Mario El Mentar apparatus, Organic Microanalysis Unit, and the results were within the accepted range (± 0.20) of the calculated values. Follow up of the reactions and checking the purity of the compounds was made by TLC on silica gel-precoated aluminum sheets (Type 60 F254; Merck, Darmstadt, Germany). Compounds **1** was prepared according to reported method (m.p 140-141 ˚C and *lit.* m.p 139–141˚C [24]).

*6-Mercapto-1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-1H-pyrazolo [3,4-]pyrimidin--one (**2**)*

In a round flask containing 0.01 mol of potassium isothiocyanate in 30 mL of boiling ethanol, an equimolar amount of methyl 5-amino-1-(9-methyl-5,6-dihydro-naphtho[1',2':4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazole-4-carboxylate (**1**) was added and the reaction mixture was left to reflux for 30 minutes and filtered off, then 15 mL of hydrochloric acid were added to the formed filtrate with stirring till complete precipitation. The reaction mixture was left to boil for 15 minutes. The formed precipitate was collected by filtration, washed several times with hot water, then recrystallized from dioxane to give the title compound **2**. Pale yellow fine crystals; (Yield 88%); m.p. 136-137^{o}C; IR (KBr) υ cm^{-1}: 3103.87 (-NH), 2654.53 (-SH), 1699.94 (N-C=O), 1616.06 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.39 (s, 3H, CH_3), 2.52 (s, 1H, SH), 2.83-2.92 (m, 4H, $2CH_2$), 7.16-7.26 (2m, 4H, 3Ar-H+ H3-pyrazole), 8.40 (d, 1H, *J* = 7.65 Hz, Ar-H), 12.49 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 20.44 (CH_3), 23.37 (CH_2), 29.24 (CH_2), 118.35-158.04 (15Ar-C), 164.12 (C=O); MS m/z (%): 420 (M^++2, 45.5), 104 (9.1), 103 (45.5), 76 (45.5), 64 (100); Anal. calcd. for $C_{20}H_{14}N_6OS_2$ (418.49) (%): C, 57.40; H, 3.37; N, 20.08; S, 15.32. Found (%): C, 57.19; H, 3.21; N, 19.99; S, 15.11.

*6-Methylthio-1-(9-methyl-5,6-dihydronaphtho-[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-1H-pyrazolo-[3,4-d]pyrimidin-4-one (**3**)*

Compound **2** (1 mmol) in alcoholic potassium hydroxide, was treated with an equivalent amount of methyl iodide (1 mmol) and the reaction mixture was refluxed for 3 h. The formed precipitate was filtered off, dried and recrystallized from ethanol to give the title compound **3**. Pale yellow powder; (Yield 81%); m.p. 214-216^{o}C; IR (KBr) υ cm-1: 3429 (NH), 1663 (NH-C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 2.36 (s, 3H, S-CH_3), 2.54 (s, 3H, CH_3), 2.80-2.90 (m, 4H, $2CH_2$), 7.14-7.25 (2m, 4H, 3Ar-H+ H3-pyrazole), 8.29 (d, 1H, *J* = 7.8 Hz, Ar-H), 8.41 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 18.53 (S-CH_3), 20.53 (CH_3), 22.93 (CH_2), 29.26 (CH_2), 117.40-158.11 (14Ar-C), 162.41 (C-S), 164.60 (C=O); MS, m/z (%): 432.15 (M^+, 0.16), 431.10 (1.26), 363 (100), 294 (39.96), 288.05 (54.11); 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 (%): 58.42; H,

3.91; N, 19.30; S, 14.97.

*General procedure for the Synthesis of **4**, **5**, **6**, **7** and **8**:*
To a solution of sodium ethoxide (0.01 mol Na metal in dry ethanol), compound **2** (0.01 mol) was added, and then the reaction mixture was stirred at room temperature for about 1h. 2-Chloroethyl methyl ether, chloroacetaldehyde dimethyl-acetal, 2-chloroehanol, 2-(2-chloroethoxy)-ethanol or epichlorohydrine (0.01mol) was added and the reaction mixture was stirred at 70°C for 3-4h, respectively. The reaction mixtures were evaporated under reduced pressure and the residue were crystallized using ethanol to give the title compounds **4**, **5**, **6**, **7** and **8** respectively.

*6-[(Methoxyethyl)thio]-1-(9-methyl-5,6-dihydro-naphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-5-hydro-4H-pyrazolo[3,4-d]pyrimidine-4-one (**4**)*
Yellow crystals; (Yield 58%); m.p. 189-190^{o}C; IR (KBr) υ cm^{-1}: 3433.64 (NH), 1659.45 (N-C=O), 1594.84 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.20 (s, 3H, CH_3), 2.46 (s, 3H, OCH_3), 2.74-2.89 (2m, 4H, $2CH_2$), 3.25 (m, 2H, -SCH_2), 4.53 (m, 2H, -CH_2-O), 7.20-7.28 (2m, 4H, 3Ar-H+H-pyrazole), 7.88 (d, 1H, *J* = 7.6 *Hz*, Ar-H), 11.60 (s, 1H, NH, D_2O exchangeable); MS, *m/z* (%): 478.10 (M^++2, 9.30), 477.10 (M^++1, 14.52), 476.10 (M^+, 9.30), 461.10 (M^+-CH_3, 10.11), 447.10 (M^+-CH_2CH_3, 11.58), 432.10 (M^+-OCH_2CH_3, 15.99), 316.10 (10.11), 251.10 (3.92), 115.05 (100.00), 91.05 (-$SCH_2OCH_2CH_3$, 17.62); Anal. calcd. for $C_{23}H_{20}N_6O_2S_2$ (476.57) (%): C, 57.96; H, 4.23; N, 17.63; S, 13.46. Found (%): C, 57.77; H, 4.35; N, 17.72; S, 13.35.

*6-[(2,2-Dimethoxyethyl)thio]-1-(9-methyl-5,6-dihydro-naphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-5-hydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**5**)*
Pale yellow powder; (Yield 81%); m.p. 136-137^{o}C; IR (KBr) υ cm^{-1}: 3437.49 (NH), 1658.48 (NH-C=O), 1593.88 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.51 (s, 3H, CH_3), 2.83-2.90 (2m, 4H, $2CH_2$), 3.32 (s, 6H, 2-O-CH_3), 3.83 (d, 2H, S-CH_2), 4.50 (t, 1H, S-CH_2-CH), 7.15-7.26 (2m, 4H, 3Ar-H+H-pyrazole), 8.41 (d, 1H, *J* = 8 *Hz*, Ar-H), 12.45 (s, 1H, NH, D_2O exchangeable); MS, *m/z* (%): 506.10 (M^+, 28.06), 491.10 (M^+-CH_3, 45.41), 475.10 (M^+-OCH_3, 32.14), 444.10 (M^+-2OCH_3, 8.16), 195.00 (100). Anal. calcd. for $C_{24}H_{22}N_6O_3S_2$ (506.60) (%): C, 56.90; H, 4.38; N, 16.59; S, 12.66. Found (%): C, 56.75; H, 4.49; N, 16.39; S, 12.49.

*6-[(2-Hydroxyethyl)thio]-1-(9-methyl-5,6-dihydro-naphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-5-hydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**6**)*
Pale brown oil; (Yield 61%); IR υ cm^{-1}: 3416.28 (-NH&-OH), 1659.45 (O=C-NH), 1594.84 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.46 (s, 3H, CH_3), 2.82-2.86 (2m, 4H, $2CH_2$), 3.55 (d, 2H, S-CH_2), 4.34 (m, 2H, -CH_2-OH), 5.20 (br, 1H, OH, D_2O exchangeable), 7.20-7.23 (2m, 4H, 3Ar-H+H-pyrazole); 8.35 (d, 1H, *J*=7.5 *Hz*, Ar-H), 12.40 (s, 1H, NH, D_2O exchangeable); MS, *m/z* (%): 463.00 (M^++1, 34.62), 462.00 (M^+, 46.79), 430.00 (M^+- CH_2OH, 35.26), 417.00 (M^+-CH_2CH_2OH, 58.97), 385.00 (M^+- SCH_2CH_2OH, 39.74), 316.00 (40.38), 268.00 (100), 251.00 (50.00); Anal. calcd. for $C_{22}H_{18}N_6O_2S_2$ (462.54) (%): C, 57.13; H, 3.92; N, 18.17; S, 13.86. Found (%): C, 56.98; H, 4.03; N, 18.37; S, 13.69.

*6-([2-(2-Hydroxyethoxy)ethyl]thio)-1-(9-methyl-5,6-dihydro-naphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-5-hydro-4H-pyrazolo[3,4-d]pyrimidine-4-one (**7**)*
Pale brown powder; (Yield 54%); m.p. 189-190^{o}C; IR (KBr) υ cm^{-1}: 3402.78 broad (OH), 1627.63 (N-C=O), 1405.85 (C-S); ^{1}H NMR (DMSO-d_6) δ ppm: 2.38 (s, 3H, CH_3), 2.83-2.90 (2m, 4H, $2CH_2$), 2.95 (m, 2H, S-CH_2), 3.55-3.73 (2m, 4H, -CH_2-CH_2-OH), 3.85 (m, 2H, S-CH_2-CH_2-O) 5.32 (br, 1H, OH, D_2O exchangeable), 7.15-7.26 (2m, 4H, 3Ar-H+H-pyrazole), 8.41 (d, 1H, *J* = 8 *Hz*, Ar-H), 12.47 (s, 1H, NH, D_2O exchangeable); MS, *m/z* (%): 505.30 (M^+-1, 0.43), 474.30 (M^+-CH_2OH]$^+$, 0.06), 110.85 (40.03), 71.05 (70.08); Anal. calcd. for $C_{24}H_{22}N_6O_3S_2$ (506.60) (%): C, 56.90; H, 4.38; N, 16.59; S, 12.66. Found (%): C, 56.74; H, 4.27; N, 16.79; S, 12.79.

*6-[(2,2-Dihydroxyethyl)thio]-1-(9-methyl-5,6-dihydro-naphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-5-hydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**8**)*
Pale white fine crystals; (Yield 49%); m.p. 189-190^{o}C; IR (KBr) υ cm^{-1}: 3399.89 sharp (OH), 1655.59 (N-C=O), 1553.38 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.51 (s, 3H, CH_3), 2.68 (m, 1H, Ha, S-CH_2-), 2.85-2.91 (2m, 4H, $2CH_2$), 3.46 (d, 1H, *J* = 4 *Hz*, Hb, S-CH_2-), 3.88 (m, 1H, Ha, N-CH_2-), 4.35 (d, 1H, *J*=12 *Hz*, Hb, N-CH_2-), 4.84 (m, 1H, CH-OH), 5.11 (d, 1H, *J*=4 *Hz*, OH, D_2O exchangeable), 7.17-7.28 (2m, 4H, 3Ar-H), 7.37 (s, 1H, H3-pyrazole), 8.34 (d, 1H, *J*=8 *Hz*, Ar-H); ^{13}C NMR (DMSO-d_6) δ ppm: 20.55 (CH_2), 23.46 (CH_2), 29.33 (CH_3), 48.02 (S-CH_2), 64.18 (N-CH_2), 68.74 (CH-OH), 117.86-157.67 (16 sp^2-C), 162.59 (C=O); MS, *m/z* (%): 476.00 (M^++2, 48.06), 475.00 (M^++1, 62.79), 474.00 (M^+, 58.91), 473.00 (M^+-1, 61.24), 472.00 (M^+-2, 51.16), 459.00 (41.86), 417.00 (48.84), 386.00 (79.84), 50 (100); Anal. calcd. for $C_{23}H_{18}N_6O_2S_2$ (474.56) (%): C, 58.21; H, 3.82; N, 17.71; S, 13.51. Found (%): C, 58.08; H, 3.71; N, 17.87; S, 13.64.

*[(1-(9-methyl-5,6-dihydronaphtho-[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-4-oxo-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio]-acetic acid (**9**)*

To a solution of compound **2** (5 mmol) in 20 mL dry alcohol an equivalent amount of chloroacetic acid was added, the reaction mixture was left to reflux for 4 h and followed with TLC until the reaction was completed. The reaction mixture was poured onto crushed ice and the formed solid was collected by filtration, washed with water and air dried to be recrystallized from dioxane to give the title compound **9**. Yellow crystals; (Yield 78%); m.p. 189-190^{o}C; IR (KBr) υ cm^{-1}: 3620.44 (broad-OH), 2953.45 (C-H aromatic-H), 2857.02 (C-H aliphatic), 1720.56 (-COOH), 1650.77 (N-C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 2.38 (s, 3H, CH_3), 2.85-2.95 (m, 4H, $2CH_2$), 4.28 (s, 2H, CH_2), 7.15-7.42 (m, 4H, 3Ar-H+H3-pyrazole), 8.40 (d, 1H, *J*=7.5 *Hz*, Ar-H), 10.40 (s, 1H, NH, D_2O exchangeable), 12.42 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 20.53 (CH_3), 23.38 (CH_2), 29.25 (CH_2), 41.45 (CH_2-COOH), 118.31-154.82 (sp^2 Carbons), 158.12 (C-S), 164.58 (N-C=O), 168.53 (HO-C=O); MS *m/z* (%): 476.00 (M^+, 0.43), 292.10 (0.13), 267.05 (100), 251.00 (0.63), 91.05 (37.00); Anal. calcd. for $C_{22}H_{16}N_6O_3S_2$ (476.53) (%): C, 55.45; H, 3.38; N, 17.64; S, 13.46. Found (%): C, 55.64; H, 3.51; N, 17.53; S, 13.32.

*1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-pyrazolo[3,4-d][1,3] thiazolidino-[3,2-a]pyrimidin-4,6(7H)-dione (**10**)*

To a solution of compound **9** (2.5 mmol) in 20 mL dry alcohol was added glacial acetic acid/acetic anhydride (20/10 mL) in presence of anhydrous sodium acetate (0.5 gm), the reaction mixture was left to reflux for 2h and followed with TLC until the reaction was completed. The reaction mixture was poured onto crushed ice and the formed solid was collected by filtration, washed with water and air dried to be recrystallized from dioxane to give the title compound **10**. Pale white powder; (Yield 91%); m.p. 212-214^{o}C; IR (KBr) υ cm^{-1}: 2919.70 (C-H aromatic-H), 2855.10 (C-H aliphatic), 1659.45 (C=O), 1593.88 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 2.51 (s, 3H, CH_3), 2.85-2.95 (m, 4H, $2CH_2$), 3.92 (s, 2H, CH_2), 7.17-7.40 (m, 4H, 3Ar-H+H3-pyrazole), 8.41 (d, 1H, *J*=8 *Hz*, Ar-H); MS, *m/z* (%): 459.10 (M^++1, 1.74), 458.10 (M^+, 2.35), 430.10 (M^+-C=O, 2.61), 416.10 (2.15), 279.10 (1.51), 263.00 (100); Anal. calcd. for $C_{22}H_{14}N_6O_2S_2$ (458.51) (%): C, 57.63; H, 3.08; N, 18.33; S, 13.99. Found (%): C, 57.45; H, 3.19; N, 18.26; S, 13.90.

*7-Hydroxy-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]-thieno[2,3-d]pyrimidin-11-yl)-pyrazolo[3,4-d][1,3]-thiazolo[3,2-a]pyrimidin-4(1H)-one (**11**)*

To a solution of 2.5 mmol of compound **2** in dry dioxane 20 mL, was added an equivalent amount of chloroacetyl chloride and a catalytic amount of triethylamine (3 drops) and the reaction mixture was heated under reflux for 6h. The reaction mixture was poured onto crushed ice and the formed solid was collected by filtration and washed with hot water and ethanol, air dried and recrystallized from dioxane to give the title compound **11** in 79% yield. White powder; (Yield 79%); m.p. 230-231^{o}C; IR (KBr) υ cm^{-1}: 3260.44 (broad -OH), 2921.63 (C-H aromatic-H), 2854.13 (C-H aliphatic); ^{1}H NMR (DMSO-d_6) δ ppm: 2.50 (s, 3H, CH_3), 2.80-2.92 (2m, 4H, $2CH_2$), 7.12 (s, 1H, thiophene-H), 7.34-7.51 (2m, 4H, 3Ar-H+ H3-pyrazole), 8.02 (d, 1H, *J* = 7.6 *Hz*, Ar-H), 12.74 (s, 1H, OH, D_2O exchangeable); MS, *m/z* (%): 460.40 (M^++2, 0.64), 459.40 (M^++1, 0.61), 458.40 (M^+, 0.60), 281.25 (15.50), 71.05 (22.50), 57.05 (100); Anal. calcd. for $C_{22}H_{14}N_6O_2S_2$ (458.51) (%): C, 57.63; H, 3.08; N, 18.33; S, 13.99. Found (%): C, 57.46; H, 3.97; N, 18.47; S, 14.19.

3.2.1. Chemicals

Fetal bovine serum (FBS) and L-glutamine, were obtained from Gibco Invitrogen Company (Scotland, UK). Dulbecco's modified Eagle's (DMEM) medium was provided from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, and streptomycin were obtained from Sigma Chemical Company (Saint Louis, MO, USA). The level of uPA protein was determined using Assay Max human urokinase (uPA) ELISA kit (Assaypro, USA).

3.2.2. Cell lines and culturing

Anticancer activity screening for the tested compounds utilizing 2 different human tumor cell lines including breast cancer cell line MCF-7 and liver cancer cell line HepG2 were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 ^{o}C in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50 x 10^6 were grown in a 25 cm^2 flask in 5 ml of complete culture medium.

3.2.3. *In Vitro* cytotoxicity assay

The cytotoxicity activity was measured *in vitro* using the Sulfo-Rhodamine-B stain (SRB) assay according to the previous reported standard procedure [26]. Cells were inoculated in 96-well microtiter plate (10^4 cells/ well) for 24 h before treatment with the tested compounds to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO at 1 mg/ml immediately before use and diluted to the appropriate volume just before

addition to the cell culture. Different concentration of tested compounds and doxorubicin were added to the cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h. at 37°C and in atmosphere of 5% CO_2. After 48 h cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Table 1. The results were compared to the antiproliferative effects of the reference control doxorubicin.

3.2.3. Determination the level of uPA protein expression

The level of uPA protein expression was determined using Assay Max human urokinase (uPA) ELISA kit (Assaypro, USA) according to manufacturer's instructions. The prepared compounds as well as standard drug, doxorubicin were incubated for 48 h with MCF7 and HepG2 cells at concentration of 1/10 of the IC_{50} values of each compound which shown in Table 1. After 48 h from compounds treatment, medium was collected and centrifuged at 2000 xg for 10 min to remove cellular debris. Add 50 µl of the cell extract per well and incubate for 2 h. Wells were washed with 200 µl of wash buffer then add 50 µl of biotinylated uPA antibody to each well and incubate for 1 h at 25ºC. After washing, plates were incubated with 50 µl of streptavidin-peroxidase conjugate per well and incubate for 30 minutes then wash the microplate as described above. Add 50 µl of chromogen substrate per well and incubate for about 10 min or till the optimal blue color density develops. Add 50 µl of stop solution to each well. The color will change from blue to yellow. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately and the concentrations of uPA in the samples were determined and the percentage of uPA inhibition for each compound was calculated as compared with control cancer cells (DMSO treated).

Statistical analysis

The results are reported as Mean ± Standard error (S.E.) for at least three times experiments. Statistical differences were analyzed according to followed by one way ANOVA test followed by student's *t* test wherein the differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

2.1. Chemistry

In this respect, this work describes efficient methods for the synthesis of new compounds derived from the *β*-enaminoester of methyl 5-amino-1-(9-methyl-5,6-dihydro-naphtho[1',2':4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazole-4-carboxylate (**1**) [24] which was used as the key compound in this study and for further syntheses.

Thus, compound 6-mercapto-1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-one (**2**) was prepared by the reaction of compound **1** [24] with potassium isothiocyanate followed by alkaline hydrolysis. The structure of compound **2** was confirmed by the elemental analysis and spectral data (c.f. experimental).

Alkaline treatment of compound **2** with methyl iodide in the presence of potassium hydroxide gave the corresponding *S*-alkyl derivative 6-methylthio-1-(2-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-*d*]-pyrimidin-11-yl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-one (**3**). The ^{1}H NMR spectrum of the latter compound showed signal for the *S*-alkyl group at δ 2.36 and ^{13}C NMR spectrum showed signal at δ 18.53 (S-CH_3) which indicated that the site of attack is on the sulfur and not on the nitrogen.

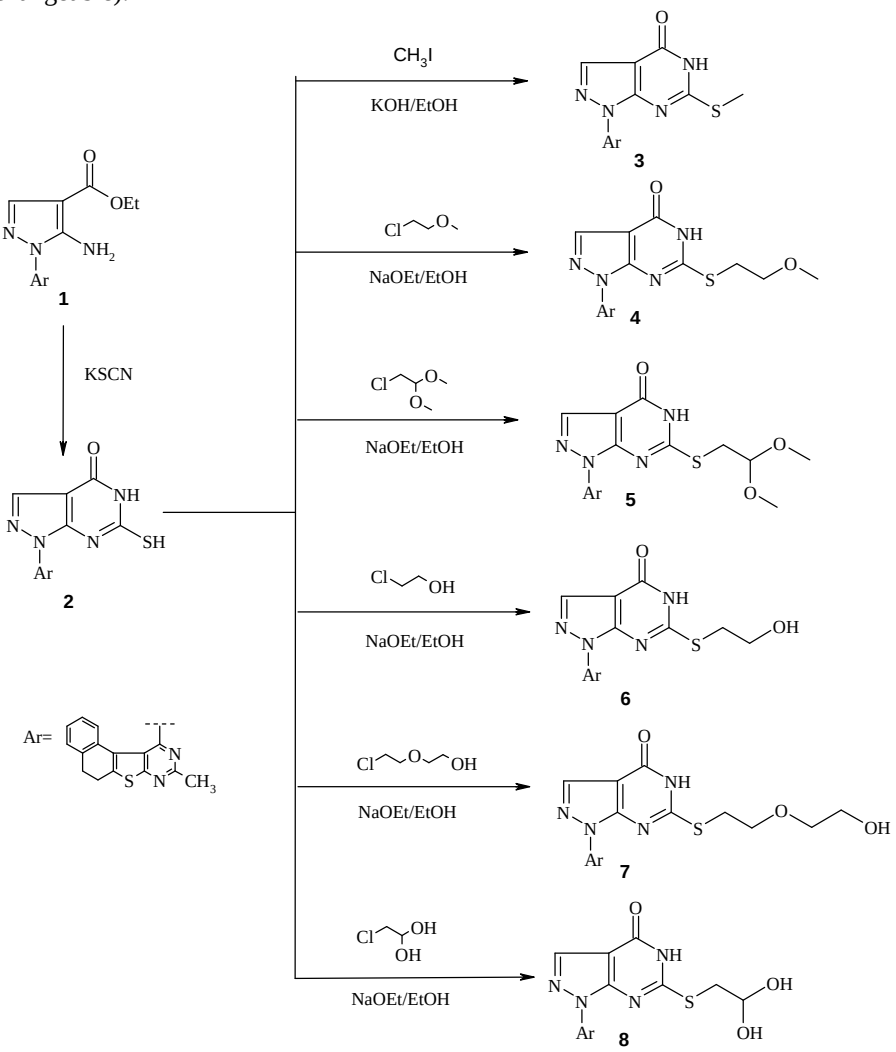
Few *S*-acyclic nucleosides of pyrazolo[3,4-*d*]pyrimidine ring system are reported in literature [8,9,17]. So, when compound **2** was treated with 2-chloroethyl methyl ether, chloracetaldehyde dimethylacetal, 2-chloroethanol, 2-(2-chloroethoxy)ethanol, and epichlorohydrin it afforded the corresponding *S*-acyclic nucleosides **4-8**, respectively (Scheme 1). The IR spectra of the latter compounds revealed the presence of the absorption bands for the C=O and N-H for each compound. In addition, the IR spectra revealed the presence of the OH absorption band of compounds **6-8**. Also, ^{13}C NMR spectra of the *S*-acyclic nucleosides **4-8** indicated the absence of the C=S group and their ^{1}H NMR spectra indicated the presence of methoxyethyl, dimethoxyethyl, hydroxyethyl, hydroxymethoxy ethyl and dihrdroxyethyl signals, respectively (cf. experimental).

Similarly, treatment of compound **2** with 2-chloroacetic acid in ethanol gave the corresponding [(1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-*d*]pyrimidin-11-yl)-4-oxo-4,5-dihydro-1*H*-pyrazolo[3,4-*d*]pyrimidin-6-yl)thio]-acetic acid (**9**). The IR spectrum of the latter compound showed the presence of 2 C=O at υ 1650.77 (N-C=O) and at

υ 1720.56 cm^{-1} (-COOH) and broad band for the -OH of the acid at υ 3620.44 cm^{-1}. In addition, the mass spectrum of compound **9** confirmed the formation of the open structure [m/z 476 (M+)].

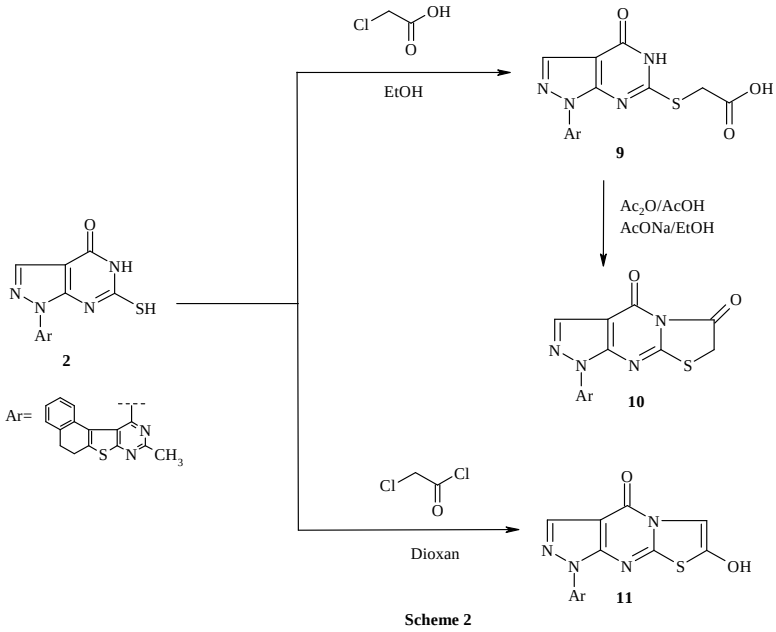
When compound **9** was treated with glacial acetic acid/acetic anhydride (20/10 mL) in the presence of anhydrous sodium acetate, it cyclized to give 1-(9-methyl-5,6-dihydronaphtho-[1',2':4,5]thieno[2,3-*d*]pyrimidin-11-yl)pyrazolo[3,4-*d*][1,3]thiazolidino[3,2-*a*]pyrimidin-4,6(7*H*)-dione (**10**) (Scheme 2). The type of cyclization to produce compound **10** is in accordance with previous reported results [25].

However, treatment of compound **2** with chloroacetyl chloride in the presence of a catalytic amount of triethylamine it gave directly the corresponding 7-hydroxy-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno-[2,3-*d*]-pyrimidin-11-yl)-pyrazolo[3,4-*d*][1,3]thiazolo[3,2-*a*]pyrimidin-4(1*H*)-one (**11**) without the isolation of the non-cyclized product. The structure of the latter compound was confirmed by different spectroscopic data where the IR revealed the presence of υ 3260.44 (broad -OH) and the ^{1}H NMR showed signal at δ 7.12 for the thiophene proton and at δ 12.74 for OH (D_2O exchangeable).



Scheme 1

Scheme 1. Synthesis of compounds 2-8



Scheme 2

Scheme 2. Synthesis of compounds 9-11

2.2. Biological activity

2.2.1. *In vitro cytotoxicity activity*

The cytotoxicity of the synthetic compounds **2-11** were tested using SRB assay as described by Skehan *et al*. [26] in breast cancer cell line MCF-7 and liver cancer cell line HepG2. For comparison, doxorubicin was also tested. The results revealed that compound **8** (IC_{50}: 2.60±0.27 and 3.75±0.44 µg/ml, respectively) exhibited similar activity to doxorubicin (IC_{50}: 2.80±0.24 and 3.75±0.35µg/ml, respectively) in both MCF-7 and HepG2. The order of cytotoxicity activity of the tested compounds was **8**, **7**, **6**, **5**, **4**, **9**, **11**, **10**, **2**, and **3** in a descending order.

The level of uPA protein expression

To identify the mechanism of action responsible for the cytotoxicity of prepared compounds **2-11** the level of uPA protein expressed in the two cell lines (breast cancer cell line MCF-7 and liver cancer cell line HepG2) were estimated quantitatively. The result revealed that the data of uPA expression were in consistent with the cytotoxicity activity. In case of breast cancer cell line MCF-7 and liver cancer cell line HepG2, the level of uPA decreased in compounds by the following percent in MCF-7 and HepG2 respectively **2 (**7, 5%**), 3 (**3, 5%**), 4 (**66, 60%**), 5 (**77, 63%), **6 (**82, 67%), **7 (**86, 83%), **8 (**90, 87%**), 9 (**60, 56%**), 10 (**9, 6%) and **11 (**54, 48%). From the results, compound **8** exhibited a good activity in MCF-7 (90%) and HepG2 (87%) similar to doxorubicin (91% and 88%, respectively) (Table 1) in both MCF-7 and HepG2. The order of uPA activity inhibition of the tested compounds was **8**, **7**, **6**, **5**, **4**, **9**, **11**, **10**, **2**, and **3** in a descending order which are in accordance with the cytotoxicity activity.

Table 1. In vitro cytotoxicity activity and the percent inhibition of uPA of the synthesized compounds on the cell lines

Compound	IC_{50} (µg/ml)		% inhibition of uPA [a]	
	MCF-7	HepG2	MCF-7	HepG2
Doxorubicin	2.80±0.24	3.90±0.37	91±3.70	88±6.36
DMSO	N.A.	N.A.	N.A.	N.A.
2	16.70±1.50	18.30±1.75	7±0.94	5±0.46
3	19.00±1.85	20.00±1.92	3±0.45	5±0.46
4	5.90±0.56	8.00±0.92	66±5.44	60±5.09
5	5.00±0.63	6.20±0.80	77±4.28	63±4.65
6	4.60±0.48	4.80±0.47	82±6.95	67±4.78
7	3.65±0.29	4.20±0.44	86±3.09	83±4.65
8	2.60±0.27	3.75±0.44	90±5.76	87±6.45
9	5.70±0.72	6.90±0.65	60±2.84	56±1.98
10	14.18±1.36	16.10±1.40	9±0.47	6±0.45
11	6.30±0.60	7.25±0.35	54±4.93	48±4.82

[a]*The percentage changes as compared with control untreated cancer cells (DMSO treated).*
N.A. is no activity

Taken together, these findings suggested that there are correlation between the cytotoxicity of the tested compounds and inhibition of the urokinase activity. The tested compounds exert anti-carcinogenic activity in MCF-7 breast and

HepG2 liver cancer cells through inhibiting the activity of urokinase enzyme which may reduce the cell proliferation and resulted in significant growth inhibitory.

CONCLUSION

In conclusion, the present results suggested that there are correlation between the cytotoxicity of the synthesized compounds **2-11** and inhibition of the urokinase activity. The tested compounds exert anti-carcinogenic activity in hepatic HepG2 and breast MCF-7 cancer cell lines through down-regulation the activity of urokinase enzyme which may reduce the cell proliferation and resulted in significant growth inhibitory, especially, compounds **8** and **7** which revealed promising activity compared to the activity of the commonly used anticancer drug, doxorubicin.

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