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Novel fused thienopyridne and pyrazolopyridine derivatives: Synthesis, characterization and cytotoxicity

Mohamed A. A. Elneairy[1], Sanaa M. Eldine[2] and Ahmed S. I. Mohamed*[3]

[1]Chemistry Department, Faculty of Science, Cairo University, Giza, Egypt
[2]Pesticides Chemistry Department, National Research Centre, Dokki, Giza, Egypt and
[3]Chemistry of Natural Compounds Department, National Research Centre, Dokki, Giza, Egypt

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ABSTRACT

*Several new of fused thienopyridine and pyrazolopyridine derivatives were synthesized via the reactions of both compounds 2- Carbohydrazide **2** and 3-aminopyrazolopyridine **14** with a variety of active reagents and chemicals. Structures were established based on elemental and spectral data studies. Some of the synthesized compounds exploited potent antitumor activity, especially the pyridopyrazolotriazine **20c** which displayed the highest activity among the tested compounds with the IC_{50} equal to 3.8µg/ml.*

Key words: Pyridothienopyrimidinone, Pyridothienotriazines, Pyridopyrazolotriazines, Pyridopyrazolopyrimidine and Anti-tumor cytotoxicity.

INTRODUCTION

Cancer is a very complex disease which affects different organs and systems of the body. Its burden is increasing across the world dramatically, and it is considered as the first leading cause of deaths in economically developed countries and the second leading cause of deaths in developing countries [1].

Synthesis of the pyridine ring system and its derivatives occupy an important place in the realm of synthetic organic chemistry, due to their therapeutic and pharmacological properties [2,3]. The pyridine ring is also an integral part of anticancer [4].

The literature review showed that thieno[2,3-b]pyridine and pyrazolo[3,4-*b*]pyridines derivatives have attracted considerable attention because of their broad pharmacological activities. For example, some of the thieno[2,3-b]pyridine derivatives exhibited anticancer [5-7], antiviral [8], anti-inflammatory [9,10] antimicrobial [11], antidiabetic [12,13], antihypertensive [14,15] and osteogenic [16] activities, in addition to treatment of CNS disorders [17], Anti-hepatocellular [18] Also, pyrazolo[3,4-*b*]pyridines are antimicrobial agents [19], inhibitors of glycogen synthase kinase-3 (GSK-3) [20] and potent antitumor agents [21,22].

Numerous derivatives of tricyclic heteroaromatic systems, containing the biologically active moiety of thieno[2,3-b]pyridine and pyrazolo[3,4-*b*]pyridine showed important pharmacological properties as anticancer activity [23-25].

The broad range of pharmacological activity of this class of compounds prompted us to synthesize new derivatives of a tricyclic system via heterocyclic compounds containing thieno[2,3-b] pyridine and pyrazolo[3,4-*b*] pyridines moiety condensed with each of pyrazole, pyrimidine, 1,2,3 triazole and imidazole to examine their antiproliferative activity *in vitro* against three human cancer cell lines.

MATERIALS AND METHODS

All melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. The IR spectra were recorded (KBr disk) on a Shimadzu FT IR-8201 PC instrument. 1H NMR spectra (300 MHz) spectra were recorded on a Varian spectrometer using DMSO-*d*6 or CDCl3 as solvent and TMS as an internal standard. Chemical shifts are 36 reported in ppm. Mass spectra were recorded on a Shemadzu GCMS-QP1000EX using inlet type at 70 ev. Elemental analyses were obtained from The Micro analytical Data Center at Cairo University, Egypt. Progress of the reactions was monitored by thin-layer chromatography (TLC) using aluminum sheets coated with silica gel F254 (Merck), viewing under a short-wavelength UV 41 lamp effected detection. All evaporations were carried out under reduced pressure at 40 oC. Ethyl-3-amino-6-methyl-4-phenyl-5-(4-methylphenylthio) thieno[2,3-b]pyridin-2-carboxylate (**1**) and 6-Methyl-4-phenyl-2-thioxo-5-(4-methylphenylthio)-1,2-dihydropyridine-3-carbonitrile (**11)** were prepared according to the literature [26].

3-Amino-6-methyl-4-phenyl-5-(4-methylphenylthio)thieno[2,3-b]pyridine-2-carbohydrazide (2)
A mixture of compounds **1** (0.01 mol) and hydrazine hydrate (0.01 mol) was refluxed for 8 hours in ethanol (30 mL) and then the solvent evaporated under reduced pressure. The resulting solid product was collected by filtration and recrystallized from ethanol.

Yellow crystals; Yield (75%); MP: 247-249 ^{o}C; IR (KBr); 3474, 3336 (NH_2), 3739 (NH), 1620 cm^{-1} (C=O); ^{1}H NMR(DMSO-d6) δ=2.21 (s, 3H, tolyl-CH_3), 2.55 (s, 3H, pyridine-CH_3), 4.14 (*br*, 2H, NH-NH_2), 5.47 (*br*, 56 2H, NH_2 at thiophene ring), 6.74-7.49 (m, 9H, Ar-H), 9.15 ppm (*br*, 1H, NH-NH_2); Anal. Calcd. for $C_{22}H_{20}N_4OS_2$ (420.55): C, 62.83; H, 4.79; N, 13.32; S, 15.25. Found: C, 62.90; H, 4.75; N, 54 13.35; S, 15.30 %.

5-methyl-3-phenyl-4(4-methylphenylthio)pyrazolo[3',4':4,5]thieno[2,3-b]pyridine-1 (2*H*)-one (3).
A solution of **2** (0.01 mole) in glacial acetic acid (25 ml) was refluxed for 6 hours; the 61 excess solvent was evaporated in vacuum. The reaction mixture was cooled and the resulting solid product was filtered off and crystallized from ethanol.

Pale yellow crystals; Yield (90%); MP: 229-231 ^{o}C; ^{1}H NMR (DMSO-d6) δ =2.21 (s, 3H, tolyl-CH_3), 2.66 (s, 3H, pyridine-CH_3), 4.14 66 (s, 1H, NH), 5.94 (s, 1H, CONH), 6.74-7.47 ppm (m, 9H, Ar-H); Anal. Calcd. for $C_{22}H_{17}N_3OS_2$ (403.52): C, 65.48; H, 4.25; N, 10.41; S 15.89. Found: C, 65.55; H, 4.20; N, 10.48; S, 15.95 %.

3-[(N,N-diacetylamino)]-2,7-dimethyl-9-phenyl-8-(4-methyl phenylthio) pyrido [3',2':4,5] thieno [3,2-d] pyrimidin-4(3*H*)-one (4)
A solution of **2** (0.01 mole) in acetic acid anhydride (25 ml) was refluxed for 6 hours; the excess solvent was evaporated in vacuum. The reaction mixture was cooled and the resulting solid product was filtered off and crystallized from ethanol.

white crystals; Yield (67%); MP: 221-222 ^{o}C; ^{1}H NMR (DMSO-d6) δ=2.02 (s, 3H, pyrimidine-CH_3), 2.27 (s, 3H, tolyl-CH_3), 2.39 (s, 6H, $(COCH_3)_2$), 2.81 (s, 3H, pridine-CH_3), 6 -7.39 ppm (m, 9H, Ar-H). Anal. Calcd. for $C_{28}H_{24}N_4O_3S_2$ (528.65): C, 63.62; H, 4.58; N, 10.60; S 12.13. Found: C, 63.68; H, 4.63; N, 75 10.65; S, 12.08.

3-amino-7-methyl-9-phenyl-8-(4-methylphenylthio)pyrido[3',2':4,5]thieno[2,3-d] [1,2,3] triazin-4 (3H) -one(5).
A stirred cold solution (0-5 oC) of the appropriate thienopyridine-2-carbohydrazide **2** (0.01 mol) in acetic acid (10 ml) and concentrated hydrochloric acid (5 ml) was treated with cold solution of sodium nitrite (0.01 mol, 0.23 gm in 5 ml) drop wise with stirring. Stirring was continued for 2 hours. The reaction mixture was then allowed to stand at room temperature for 15 min. The solid obtained was collected by filtration and crystallized.

Pale yellow crystals; Yield (60%); MP > 300 ^{o}C; ^{1}H NMR (DMSO d6) δ=2.02 (s, 2H, NH_2), 2.21(s, 3H, tolyl-CH_3), 2.69 (s, 3H, pyridine-CH_3), 6.81-7.42 ppm (m, 9H, Ar-H). Anal. Calcd. for $C_{22}H_{17}N_5OS_2$ (431.53): C, 61.23; H, 3.97; N, 16.23; S, 14.. Found: C, 61.30; H, 4.00; N, 16.19; S, 14.

3-amino-2-anilino-7-methyl-9-phenyl-8-(4-methylphenylthio)pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4-(3H)-one (6).
A solution of **2** (0.01 mol) and phenyl isothiocyanate (0.01 mol) in pyridine (30 ml) was refluxed for 8 hours; the reaction mixture was cooled and the resulting solid product was filtered off and crystallized from ethanol.
Pale yellow crystals; Yield (90%); MP: 223-224 ^{o}C; ^{1}H NMR (DMSO d6): δ=2.20 (s, 3H, tolyl-CH_3), 2.55 (s, 3H, pyridine-CH_3), 4.15 (s, 2H, NH_2), 6.74-7.56 (m, 9H, Ar-H), 9.85 ppm (s, 1H, NH-Ph). Anal. Calcd. for $C_{29}H_{23}N_5OS_2$ (521.66): C, 66.77; H, 4.44; N, 13.43; S 12.29. Found: C, 66.70; H, 4.50; N, 13.40; S, 12.30 %.

3-[(N,N-dimethylamino)methyleneamino]-7-methyl-9-phenyl-8(4-methylphenylthio)pyrido [3',2':4,5] thieno [3,2-d] pyrimidin-4(3H)-one (7).
A solution of **2** (0.01 mol) and dimethylfomamide-dimethylacetal (DMF-DMA) (0.025 mol) in dry dioxane (30 ml) was refluxed for 8 hours. The excess solvent was evaporated in vacuum. The reaction mixture was cooled and the resulting solid product was filtered off and crystallized from ethanol/dioxan.

Yellow crystals; Yield (70%); MP: 223-225 ^{o}C; ^{1}H NMR (DMSO d6) δ=2.21 (s, 3H, tolyl-CH_3), 2.64 (s, 3H, pyridine-CH_3), 3.19 (s, 1H, =*CH*-N(CH_3)$_2$), 3.56 (s, 6H, -N(*CH_3*)$_2$), 6.80-8.00 (m, 9H, Ar-H), 8.06 ppm (s, 1H, pyrimidinone-CH). Anal. Calcd. for $C_{26}H_{23}N_5OS_2$ (485.62): C, 64.30; H, 4.77; N, 14.42; S 13.21. Found: C, 64.35; H, 4.80; N, 14.45; S, 13.18 %.

3-(ethoxymethyleneamino)-7-methyl-9-phenyl-8-(4-methyl phenylthio) pyrido [3',2':4,5] thieno [3,2-d] pyrimidin-4-one (8)
A solution of **2** (0.01 mole) and triethylorthoformate (20 ml) was refluxed for 6 hours; the excess solvent was evaporated in vacuum. The reaction mixture was cooled and the resulting solid product was filtered off and crystallized from ethanol.

White crystals; Yield (60%); MP: 210-212 ^{o}C; 1H NMR (DMSO d6) δ=1.29-1.34 (t, 3H, OCH$_2$*CH_3*), 2.27 (s, 3H, tolyl-CH_3), 2.86 (s, 3H, pyridine-CH_3), 4.23-4.25 (q, 2H, O*CH_2*CH_3), 6.78-7.45 (m, 9H, Ar-H), 7.27 (s, 1H, =*CH*-OEt), 8.09 ppm (s, 1H, pyrimidinone-CH). Anal. Calcd. for $C_{26}H_{22}N_4O_2S_2$ (486.61): C, 64.17; H, 4.56; N, 11.51; S 13.18. Found: C, 64.22; H, 4.50; N, 11.55; S, 13.20.

3-amino-7-methyl-9-phenyl-8-(4-methylphenylthio)pyrido[3',2':4,5]thieno[3,2-d]pyrimidine-4(3*H*)-one (9)
A solution of **2** (0.01 mol) and formic acid (20 ml) was refluxed for 4 hours; the excess solvent was evaporated in vacuum. The reaction mixture was cooled and the resulting solid product was filtered off and crystallized from ethanol.

Yellow crystals; Yield (80%); MP: 265-267 ^{o}C; ^{1}H NMR (DMSO d6): δ=2.21 (s, 3H, tolyl-CH_3), 2.55 (s, 3H, pyridine-CH_3), 3.56 (s, 2H, NH_2), 6.75-7.39 (m, 9H, Ar-H), 7.23 ppm (s, 1H, pyrimidinone-CH). Anal. Calcd. for $C_{23}H_{18}N_4OS_2$ (430.55): C, 64.16; H, 4.21; N, 13.01; S 14.90. Found: C, 64.20; H, 4.18; N, 13.08; S, 14.96.

7-methyl-4-oxo-9-phenyl-8-(4-methylphenylthio)pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-3(4*H*)–yl-formamide(10).
A solution of **2** (0.01 mol) and formic acid (20 ml) was refluxed for 8 hours; the excess solvent was evaporated in vacuum. The reaction mixture was cooled and the resulting solid product was filtered off and crystallized from ethanol.

Yellow crystals; Yield (75%); MP: 194-196 ^{o}C; ^{1}H NMR (DMSO d6) δ=2.21 (s, 3H, tolyl-CH_3), 2.66 (s, 3H, pyridine-CH_3), 8.43 (s, 1H, CHO), 6.01 (s, 1H, NH), 6.80-7.51 (m, 9H, Ar-H), 8.16 ppm (s, 1H, pyrimidinone-CH). Anal.Calcd. for $C_{24}H_{18}N_4O_2S_2$ (458.56): C, 62.86; H, 3.96; N, 12.22; S 13.99. Found: C, 62.80; H, 4.00; N, 140 12.25; S, 14.05.

6-Methyl-2-methylthio-4-phenyl-5-(4-methylphenylthio) nicotinonitrile (12)
A mixture of both compound **11** (0.01 mole) and iodomethane (0.015 mole) in methanolic sodium methoxide was heated under reflux for 2 hours then cooled. The solid formed was collected by filtration. Crystallized from ethanol as white crystals (60%); MP: 221-222 ^{o}C; ^{1}H NMR (DMSO d6) δ= 1.53 (s, 3H, tolyl-CH_3), 2.68 (s, 3H, pyridine-CH_3), 2.74 (s, 3H, SCH_3), 7.49-7.58 ppm (m, 9H, ArH's), Anal calcd. for $C_{21}H_{18}N_2S_2$ (362.5): C, 69.58; H, 5.00; N, 7.73; S, 17.69. found: C, 69.60; H, 5.02; N, 7.75; S, 17.70.

3-Amino-6-methyl-4-phenyl-5-(4-methylphenylthio)-1H-pyrazolo[3,4-b]pyridine (14):
A solution of compound **12** (0.01 mole), hydrazine hydrate (10 ml) and ethanol (20 ml) was heated under reflux for 10 hours. Excess solvent was evaporated in vacuum (to the 1/3 of its original volume) and cooled. The solid formed was collected by filtration. Other method, A solution of each of **1** (0.01 mole), hydrazine hydrate (10 ml) and ethanol (20 ml) was heated under reflux for 10 hours. Excess solvent was evaporated in vacuum (to the 1/3 of its original volume) and cooled. The solid formed was collected by filtration. Crystallized from ethanol as yellow crystals (40%); MP:320-322 ^{o}C; IR (KBr); 3289, 3182 (NH_2) and 3461 cm^{-}1(NH); 1H NMR (DMSO d6) δ=2.20 (s, 3H, tolyl-CH_3), 2.55 (s, 3H, pyridine-CH_3), 4.13 (s, 2H, NH_2), 6.74 (s, 1H, NH) and 6.76-7.45 ppm (m, 9H, ArH's), Anal calcd. for $C_{20}H_{18}N_4S$ (346.5): C, 69.34; H, 5.24; N, 7.73; S, 16.17. found: C, 69.33; H, 5.25; N, 16.20; S, 9.25.

3-Amino-6-methyl-4-phenyl-5-(4-methylphenyl sulfinyl)-1H-pyrazolo [3, 4-b] pyridine (15):
A mixture of each compound **14** (1 mmol) and the oxidant of AlCC-SiO_2 (3-4 mmol) was prepared in chloroform (15ml) the mixture was stirred at room temperature for 5 hours, then the mixture was filtrated and the residue was washed thoroughly with chloroform (10 ml), evaporation of the solvent gave the corresponding sulfoxide (**15**), Then the product crystallized from ethanol as yellow crystals (50%); MP:320-322 ^oC; mass: M^+ 362 (0.05%) 346 (58.58%), 331 (100%), 316 (5.75%), 299 (2.11%), 290 (4.5.9%), 253 (3%), 239 (5.3%), 197 (2.76%), 140 (6.64%) and 77 (7.52%). Anal calcd. for $C_{20}H_{18}N_4OS$ (362): C, 66.28; H, 5.01; N, 15.46; S, 8.85. found: C, 69.30; H, 5.03; N, 15.48; S, 8.87.

3-Amino-6-methyl-4-phenyl-5-(4-methylphenyl sulfonyl)-1H-pyrazolo [3,4-b]pyridine (16)
A solution of compound **14** (1 mmol) in (15 ml) glacial acetic acid and added 1-2 ml from hydrogen peroxide (50%). The reaction was stirred for 20 hours at room temperature and then poured onto water ice. The product so formed was collected by filtration, gave the corresponding sulfone (**16)**. Then the product crystallized from ethanol as yellow crystals (55%); MP:262-264 ^oC; mass: M^+ 378 (5.79%), 375 (68.97%), 343 (36.58%), 328 (25.29%), 297 (19.42), 283 (11.97%), 239 (11.3%), 211 (12%), 140 (23.91%), 139 (86.3%) and 91 (100%). Anal calcd. for $C_{20}H_{18}N_4O_2S$ (378): C, 63.47; H, 4.79; N, 14.80; S, 8.47. found: C, 63.48; H, 4.81; N, 14.82; S, 8.48.

3-Chlorodiazinyl-6-methyl-4-phenyl-5-(4-methylphenylthio)-1H-pyrazolo[3,4-b] pyridine (17):
A stirred solution (0-5 ^oC) of the appropriate pyrazolo[2,3-*b*]pyridine **14** (0.01 mole) in acetic acid (10 ml) and concentrated hydrochloric acid (5 ml) was treated with a cold solution of sodium nitrite (0.23 gm in 5 ml of cold water) during 5 min. Stirring was continued for 2 h at 0-5 ^oC; the solid so formed was collected by filtration. Crystallized from ethanol as brown crystals (70%); decompose a t MP: 142-4^oC; IR (KBr); 3445 (NH), 2118 cm^{-1} (N≡N). Anal calcd. for $C_{19}H_{15}ClN_5S$ (380.8): C, 59.92; H, 3.97; N, 18.39; S, 8.42. found: C, 59.94; H, 3.98; N, 18.38; S, 8.41.

6-Methyl-4-phenyl-5-(4-methylphenylthio)-1H-pyrazolo[3,4-b]pyridin-3-ol (18):
A solution of **17** (0.01 mole, of each), hydrochloric acid (4 ml) and ethanol 95% (20 ml) was heated under reflux for 2 hours, the excess solvent was evaporated in vacuum to the 1/3 of its original volume and cooled . The solid was collected by filtration, dried and crystallized from the proper solvent. Crystallized from ethanol as brown crystals (70%); MP: 257-260 ^oC; IR (KBr); 3429 (OH), 3186 cm^{-1} (NH). Anal calcd. for $C_{19}H_{16}N_3OS$ (334.4): C, 68.24; H, 4.82; N, 12.57; S, 9.59. found: C, 68.25; H, 4.83; N, 12.55; S, 9.60.

Synthesis of pyridopyrazolo-1,2,4-triazine derivatives 20a,b.
(General method):
A stirred solution (0-5 ^oC) of malonitrile or cyanothioacetamid (0.01 mole) and sodium acetate trihydrate (0.15 mole) in ethanol absolute (20 ml) was treated with a cold solution **17** (0.01 mole) in ethanol absolute (10ml) during 5 min. Stirring was continued for 2 h at room temp the products were poured on cold water and then solid product formed was collected by filtration and then crystallized from the proper solvent to give **20a** or **20b** respectively.

4-Amino-8-methyl-10-phenyl-9-(4-methylphenylthio)pyrido [2′,3′:3,4]pyrazolo[5,1-c][1,2,4]-triazine-3-carbonitrile (20a):
Crystallized from ethanol and dioxan as golden yellow crystals (90%); MP:265-268^oC; IR (KBr); 3302, 3432 (NH_2), 2227 cm^{-1} (CN); ^{1}H NMR (DMSO d6) δ= 2.21 (s, 3H, tolyl-CH_3), 2.72 (s, 3H, pyridine-CH_3), 6.82-7.44 (m, 9H, ArH's), 9.59 ppm (br, 2H, NH_2). Anal calcd. for $C_{23}H_{17}N_7S$ (423): C, 65.23; H, 4.05; N, 23.15; S, 7.57. found: C, 65.25; H, 4.07; N, 23.13; S, 7.56.

4-Amino-8-methyl-10-phenyl-9-(4-methylphenylthio)-3-thiocarbamoyl pyrido[2′,3′:3,4]-pyrazolo [5,1-c][1,2,4]triazine (20b):
Crystallized from ethanol and dioxan as yellow crystals (85%); MP > 340 ^oC; IR (KBr); 3300, 3442 (NH_2), 1582 cm^{-1} (C=S); ^{1}H NMR (DMSO d6) δ = 2.21 (s, 3H, tolyl-CH_3), 2.72 (s, 3H, pyridine-CH_3), 3.56 (br, 2H, NH_2) 6.84-7.46 (m, 9H, ArH's), 10.30 ppm (br, 2H, CS-$\underline{NH_2}$). Anal calcd. for $C_{23}H_{19}N_7S_2$ (457): C, 60.37; H, 4.19; N, 21.43; S, 14.02. found: C, 65.39; H, 4.20; N, 21.45; S, 14.01.

ethyl 2-cyano-2-[(6-methyl-4-phenyl-5(4-methylphenylthio)-2H-pyrazolo [3,4-b] pyridine-3-yl) hydrazono] ethanoate(21):
A stirred solution (0-5 ^oC) of the ethylcyanoacetate (0.01 mole) and sodium acetate trihydrate (0.15 mole) in ethanol absolute (20 ml) was treated with a cold solution of the compound **17** (0.01 mole) in ethanol absolute (10ml) during 5 min. Stirring was continued for 2 h at room temp. The mixture was poured on cold water and then solid product formed was collected by filtration. Crystallized from ethanol and dioxan as pale green crystals (70%); MP: 292-294^oC; IR (KBr); 3396 (NH) , (1691C=O), 2221 cm^{-1} (CN); ^{1}H NMR (DMSO d6) δ = 1.27-1.38 (t, 3H, $OCH_2\underline{CH_3}$), 2.20 (s, 3H, tolyl-CH_3), 2.50 (s, 3H, pyridine-CH_3), 4.09-4.20 (q, 2H, $O\underline{CH_2}CH_3$), 6.75-7.46 (m, 9H, ArH's) , 12.14 (s, 1H, CH-CN), 13.84 ppm (s, 1H, pyrazolo

ring-NH), Anal calcd. for $C_{25}H_{22}N_6O_2S$ (470.5): C, 63.81; H, 4.71; N, 17.86; S, 6.81. found: C, 63.82; H, 4.72; N, 17.90; S, 6.80.

Ethyl 4-Amino-10-phenyl-8-methyl-9-(4-methylphenylthio)pyrido[2′,3′:3,4]-pyrazolo[5,1-c] [1,2,4] triazine-3-carboxylate (20c):

A solution of compound **21** (0.01 mole) in absolute ethanol (30 ml) containing a catalytic amount of piperdine (0.4 ml) was heated under reflux for 5 hours. The product so formed was collected by filtration. Crystallized from ethanol and dioxan as yellow crystals (70%); MP: 300-302^{o}C; IR (KBr); 3262, 3394 (NH_2) , 1693 cm^{-1} (C=O); ^{1}H NMR (DMSO d6) δ = 1.32-1.37 (t, 3H, $OCH_2\underline{CH_3}$), 2.20 (s, 3H, tolyl-CH_3), 2.71 (s, 3H, pyridine-CH_3), 4.41-4.43 (q, 2H, $O\underline{CH_2}CH_3$), 6.82-7.45 (m, 9H, ArH's), 9.10 ppm (br, 2H, NH_2). Anal calcd. for $C_{25}H_{22}N_6O_2S$ (470.5): C, 63.81; H, 4.71; N, 17.86; S, 6.81. found: C, 63.83; H, 4.70; N, 17.89; S, 6.80.

2,4,8-trimethyl-10-phenyl-9(4-methylphenylthio)pyrido[3′,2:4,5]pyrazolo-[2,3-b] pyrimidine(22)

A solution of compound **14** (0.01 mole) and acetylaceton (5ml) in acetic acid glacial (20 ml) was heated under reflux for 6hours, the excess solvent was evaporated in vacuum to the 1/3 of its original volume and cooled . The solid was collected by filtration. Crystallized from ethanol as yellow crystals (70%); MP: 240-243^{o}C; ^{1}H NMR (DMSO d6) δ = 2.20 (s, 3H, tolyl-CH_3), 2.39 (d, 3H, pyrimidine- CH_3), 2.68 (s, 3H, pyridine-CH_3), 2.85 (d, 3H, CH_3–N), 7.38-7.44 (m, 1H, pyrimidine-CH), 6.78-7.04 ppm (m, 9H, ArH's). Anal calcd. for $C_{25}H_{22}N_4S$ (410.53): C, 73.14; H, 5.40; N, 13.65; S, 7.81. found: C, 73.15; H, 5.41; N, 13.67; S, 7.80.

2-Amino-7-methyl-9-phenyl-8(4-methylphenylthio)imidazo[1′,2′:1,5]pyrazolo[3,4-b]pyridine (24)

Compound **24** produced from the reaction of compound **14** **(**0.01 mole**)** with either chloroacetonitrile (0.01 mole) or chloroacetamide (0.01mole) in acetic acid glacial (20 ml) was heated under reflux for 6 hours, the excess solvent was evaporated in vacuum to the 1/3 of its original volume and cooled . The solid was collected by filtration. Crystallized from ethanol and dioxan as white crystals (80%); MP: 260-262^{o}C; IR (KBr); 3289, 3462 (NH_2), 1641 cm^{-1} (C=N); ^{1}H NMR (DMSO d6) δ = 2.20 (s, 3H, tolyl-CH_3), 2.61 (s, 3H, pyridine-CH_3), 3.56 (s, 2H, CH_2, $C_{(3)}$-H), 4.14 (s, 2H, NH_2), 6.72-7.45 ppm (m, 9H, ArH's). Anal calcd. for $C_{22}H_{19}N_5S$ (385.4): C, 68.55; H, 4.97; N, 18.17; S, 8.32. found: C, 68.53; H, 4.98; N, 18.19; S, 8.33.

4.2. Anti-tumor cytotoxicity bioassay in vitro

HepG2 (liver carcinoma cell line), MCF7 (breast carcinoma cell line) and HCT (colon carcinoma cell line) were obtained from the Pharmacology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. Cells were maintained in DMEM medium with 10% foetal calf serum, sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin at 37^{o}C and 5% CO_2. Potentail cytotoxicity of ***20C, 24, 22, 4*** and ***8*** were tested using the method of Skehan et al [27]. Briefly, 10^4 cells/well were plated onto 96-well dishes overnight before the treatment with the tested compounds to allow the attachment of cells to the wall of the plate. Different concentrations of each tested compound(0.1, 2.5, 5, 10 µg/ml) were added to the cell monolayer; triplicate wells were used for each individual dose. Monolayer cells were incubated with the tested agent(s) for 48h at 37^{o}C and 5% CO_2. At the end of the incubation period, the cells were fixed and stained with sulforhodamine **B** dissolved in acetic acid. Unbound stain was removed by washing four times with 1% acetic acid and the protein bound dye was extracted with tris EDTA buffer. Absorbance was measured in an ELISA reader. The relation between surviving fraction and compound concentration was plotted to get the survival curve of each tumor cell line and IC_{50}. The concentration of an agent that causes a 50% growth inhibition for each tested agent using each cell line was obtained from the survival curve.

RESULTS AND DISCUSSION

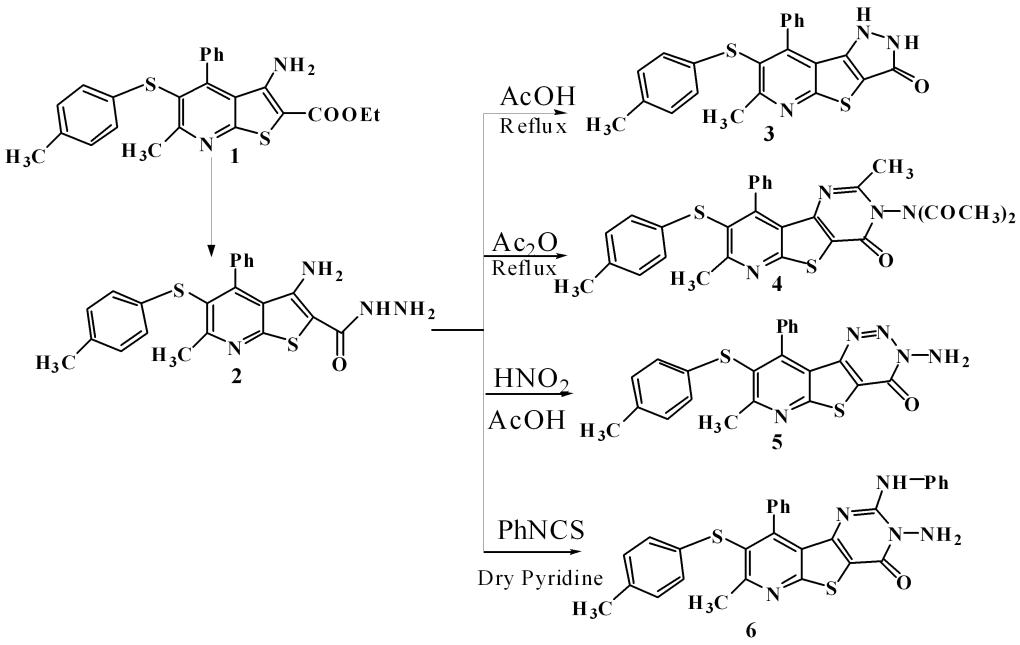
Chemistry

The synthetic procedures adopted to obtain the target compounds are depicted in Schemes **1-5**. The starting compound, ethyl-3-amino-6-methyl-4-phenyl-5-(4- methylphenylthio) thieno [2,3-*b*]pyridin-2-carboxylate (**1**), was prepared according to the previously reported procedure [26]. 3-Amino-6-methyl-4-phenyl-5-(4-methylphenylthio)thieno[2,3-*b*]pyridine-2-carbohydrazide **2** was synthesized *via* the reaction of **1** with hydrazine hydrate in refluxing ethanol. The structure of **2** was established through different spectroscopic techniques and elemental analysis data. The ^{1}H NMR spectrum of **2** showed signals at δ= 2.55 (s, 3H, pyridine-CH_3), 4.14 (*br*, 2H, NH-NH_2), 5.47 (*br*, 2H, thiophene-NH_2) and 9.15 (*br*, 1H, NH-NH_2). Also, its IR spectrum (KBr/cm^{-1}) revealed absorptions bands due to two NH_2 (3474, 3336), one NH (3739) and C=O (1620). When compound **2** was refluxed in glacial acetic acid, it can be cyclized to 5-methyl-3-phenyl-4(4-methylphenylthio) pyrazolo [3′,4′:4,5] thieno[2,3-b]pyridine-1(2*H*)-one (**3**), which was established on the basis of its elemental analysis and spectral data. ^{1}H-NMR (ppm) spectrum proved the presence of signals of two NH groups δ= 4.14 (s, 1H, NH), 5.94 (s, 1H, CONH).

Refluxing of compound **2** in acetic anhydride afforded a product that was identified as 3-[(N,N-Diacetylamino)]-2,7-dimethyl-9-phenyl-8(4-methyl phenylthio) pyrido [3′,2′:4,5] thieno [3,2-d] pyrimidin-4(3*H*)-one (**4**). IR spectrum showed the presence of the ring -CO group (1677) and CO group at (1717) and disappearance of the bands of NH_2 and NH groups. Moreover, ^{1}H-NMR spectrum revealed the presence of signals of five CH_3 groups at δ= 2.81 (s, 3H, pyridine-CH_3), 2.39 (s, 6H, $(COCH_3)_2$).

compound **2** reacted with nitrous acid in glacial acetic acid to give a product 3-amino-7-methyl-9-phenyl-8(4-methylphenylthio)pyrido[3′,2′:4,5]thieno[2,3-d][1,2,3]triazin-4(3*H*)-one **(5)**. The^{1}H-NMR spectrum revealed the presence of one signal of NH_2 (2.02) that was lost after D_2O exchange.

compound **3** reacted with phenylisothiocyanate in pyridine to afford a reaction product formed *via* equimolecular addition of the reactants and loss of hydrogen sulfide to give a reaction product 3-amino-2-anilino-7-methyl-9-phenyl-8-(4-methylphenylthio) pyrido [3′,2′:4,5] thieno[3,2-d] pyrimidin-4-(3*H*)-one (**6).** ^{1}H-NMR spectrum revealed the presence of signals at δ= 9.85(s, 1H, NH-Ph), 4.15 (s, 2H, NH_2), 2.20 (s, 3H, tolyl-CH_3). **(Scheme 1)**

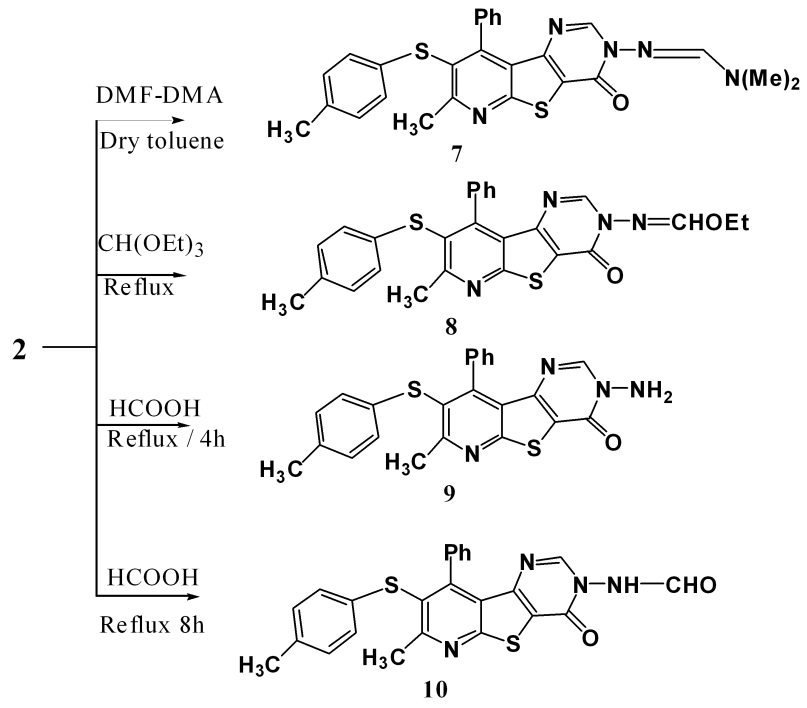


Scheme 1

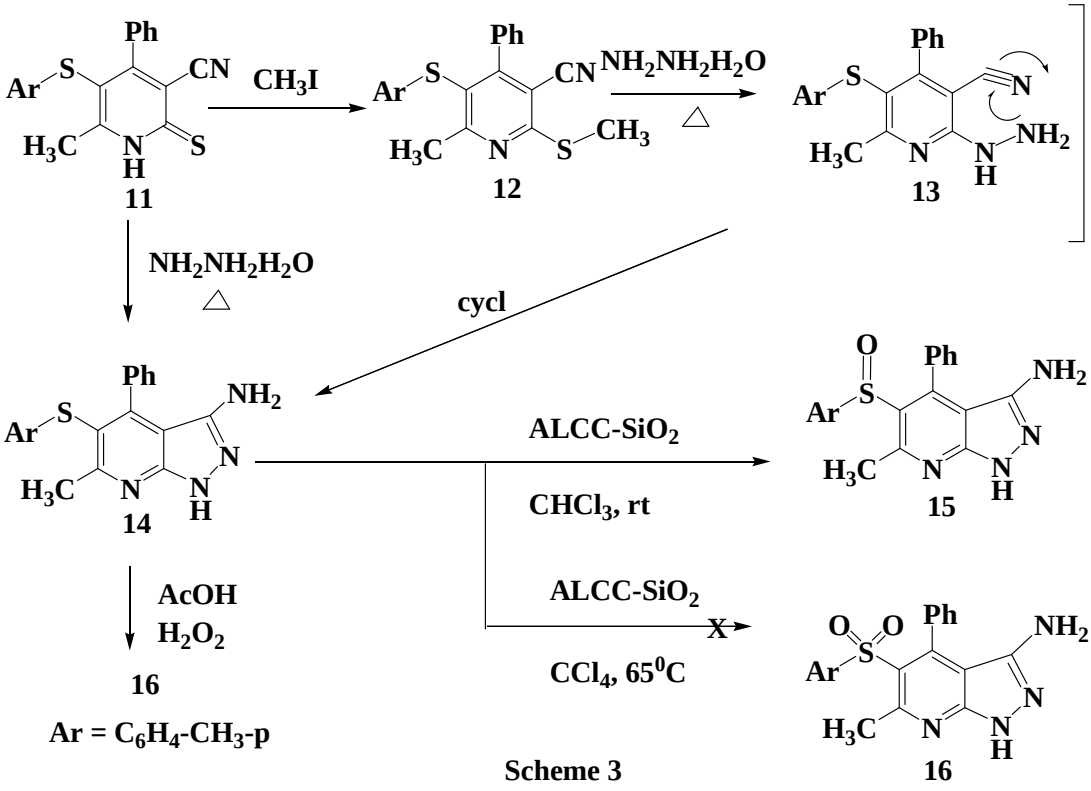
The reaction of compound **2** with dimethylformamide-dimethylacetal (DMF-DMA) in dry toluene to give a product that was identified as *3-[(N,N*-dimethylamino)methyleneamino]-7-methyl-9-phenyl 8(4methylphenylthio) pyrido [3′,2′:4,5] thieno[3,2-d]pyrimidin-4(3H)-one (**7).** The ^{1}H-NMR spectrum of this product revealed signals at δ=8.06 (s, 1H, pyrimidinone –CH), 3.56 (s, 6H, -N $(CH_3)_2$), 3.19 (s, 1H, =CH- N $(CH_3)_2$).

Refluxed a compound **2** with triethyl orthoformate. The structure of **8** was confirmed through ^{1}H NMR data that revealed the presence of the signals at δ =1.29-1.34 (t, 3H, OCH_2CH_3), 4.23-4.25 (q, 2H, OCH_2CH_3), 7.27 (s, 1H, =*CH*-OEt) and 8.09 (s, 1H, pyrimidinone-CH).

Moreover, compound **2** reacted with anhydrous formic acid to give reaction product, the nature of which depends on the reaction time. Compound, 3-amino-7-methyl-9-phenyl-8-(4- methylphenylthio)pyrido[3',2':4,5]thieno[3,2-*d*]pyrimidine-4(3*H*)-one (**9)** was obtained by the reaction of compound **2** with formic acid for 4 hours only, while, 7-methyl-4-oxo-9-phenyl-8-(4-methylphenylthio)pyrido[3′,2′:4, 5]thieno[3, 2-d]pyrimidin-3(4H)-yl-formamide (**10)**. was obtained by the reaction of compound **2** with formic acid for 8 hours. The structure of compounds **9** and **10** were established on the basis of their elemental analysis and spectral data. The 1H NMR (δ ppm) spectrum of compound **10** revealed the presence of signal at δ = 6.01 for NH and at 8.43 corresponding to formyl proton **(Scheme 2).**



Scheme 2



Scheme 3

Compound **11** was obtained according to our reported procedure [26], which reacted with methyl iodide afforded 6-methyl-2-methylthio-4-phenyl-5-(4-methylphenylthio) nicotinonitrile **(12)**. The ^{1}H-NMR spectrum of the product **2** showed SCH_3 protons as a singlet signal at δ = 2.74 ppm. When, compound 1**2** was refluxed with hydrazine hydrate afforded 3-amino-6-methyl-4-phenyl-5-(4-methyl phenyl thio)-1*H*-pyrazolo[3,4-*b*] pyridine **(14)** through cyclization of the intermediate 2-hydrazinopyridine derivatives **3**. The IR spectrum of **14** was found new bands at 3289-3182 cm^{-1} for NH_2 group and disappearance of the nitrile function peak. Compound **14** was synthesized, by another route, from the reaction of **11** with hydrazine hydrate. (See experimental section).

The reported biological activities of sulfones and sulfoxides [28,29] promted our interest to synthesis a member of these derivatives utilizing compound **14** by treatment with alanine, chlorochromic acid and silica gel (AlCC-SiO_2) [30] in chloroform at room temperature to give compound 1**5**. Its mass spectrum showed ion beaks at m/e = 362 (0.05%) consistent with the molecular ion for 1**5**.

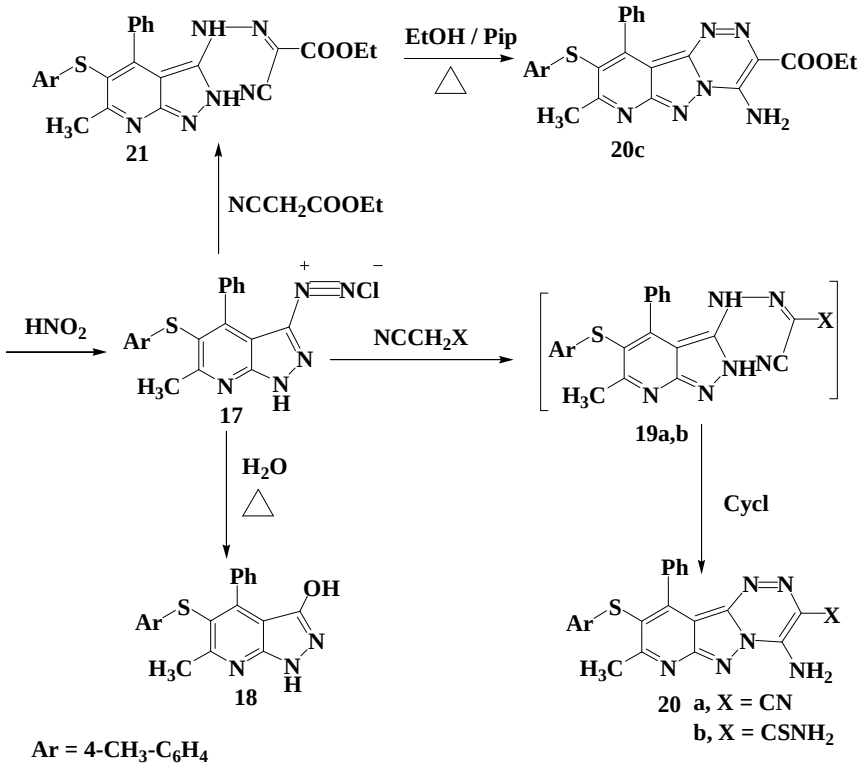
Furthermore, compound **14** reacted with hydrogen peroxide in glacial acetic acide [28] at room temperature with stirring for 20 hours to give **16**. Its mass spectrum showed ion beaks at m/e = 378 (5.79%) consistent with the molecular ion for **16**. **(Scheme 3)**

On the other hand, treatment of compound **14** with nitrous acid led to the formation of a reaction product 3-chlorodiazinyl-6-methyl-4-phenyl-5-(4-methylphenylthio)-1*H*-pyrazolo[3,4-*b*] pyridine **(17).** The IR spectrum of the isolated product was founded free from the absorption bands of the NH_2 group and showed NH (3445cm^{-1}), N≡N (2118 cm^{-1}). Compound **17** could be converted into the corresponding 6-methyl-4-phenyl-5-(4-methylphenylthio)-1*H*-pyrazolo [3,4-*b*] pyridin-3-ol **(18)** via boiling compound **17** in aqueous solution for 2 hours. The IR spectrum of compound **18** proved the presence of NH (3186 cm^{-1}) , new OH (3429 cm^{-1}) and disappear of N≡N group.

Incorporation of various functionally-substituted nitrogen heterocyclic ring into pyrazolo[3,4-b] pyridine structure, was achieved by treating **17** with different reagents. Thus, treatment of **17** with malonitrile and cyanothioacetamid under basic conditions afforded 4-amino-8-methyl-10-phenyl-9-(4-methyl phenyl thio) pyrido [2′,3′:3,4] pyrazolo[5,1-*c*][1,2,4]triazine-3-carbonitrile **(20a)** and 4-amino-8-methyl-10-phenyl-9-(4-methylphenylthio)-3-thiocarbamoylpyrido[2′,3′:3,4]pyrazolo[5,1-*c*] [1,2,4] triazine **(20b)**, respectively

In IR spectrum of compound **20a** the absorption bands at (3302, 3432 cm^{-1}) correspond to NH_2 and (2227 cm^{-1}) for CN group, Besides, the normal signals that correspond to the different protons of aromatic moieties in ^{1}H NMR spectrum signals at δ = 9.59 (br, 2H, NH_2).

Also, IR spectrum of **20b** showed the presence of absorption bands of one NH2 group (3300, 3442) and C=S (1582), ^{1}H-NMR revealed δ = 3.56 (br, 2H, NH_2) and 10.30 (br, 2H, CS-NH_2). . (See experimental section).



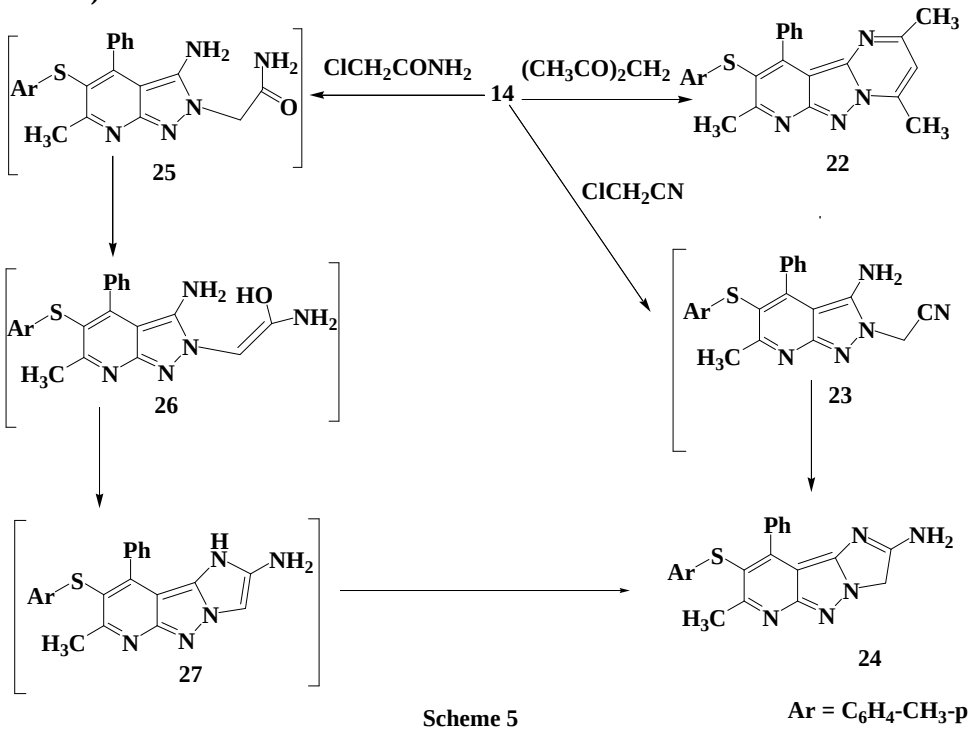
Scheme 4

In contrast to its behavior towards above reaction, compound **17** reacted with ethyl cyanoacetate to give isolable intermediate ethyl 2-cyano-2-[(6-methyl-4-phenyl-5(4-methylphenylthio)-2*H*-pyrazolo [3,4-b]pyridine-3-yl) hydrazono] ethanoate (**21).** The IR spectrum showed the absorption bands on the one NH group (3396 cm^{-1}) , ester-CO group at 1691 cm^{-1} in addition to

CN (2221 cm^{-1}), the ^{1}H-NMR revealed the signals at δ = 1.27-1.38 (t, 3H, $OCH_2\underline{CH_3}$), 4.09-4.20 (q, 2H, $O\underline{CH_2}CH_3$), 12.14 (s, 1H, CH-CN) and 13.84 (s, 1H, pyrazolo ring-NH).

A further proof for the structure of **21** came from its cyclization into the corresponding ethyl-4-amino-10-phenyl-8-methyl-9-(4-methylphenylthio)pyrido[2′,3′:3,4]-pyrazolo[5,1-c] [1,2,4]triazine-3-carboxylate (**20c)** *via* boiling its solution in ethanol in presence catalytic amount of piperidine. The structure assigned for **20c** was also based on correct elemental analysis and spectral data studies e.g IR spectra showed disappear of CN and NH group and appear NH_2 group (3262, 3394 cm^{-1}) and C=O(1693 cm^{-1}), ^{1}H-NMR showed presence of 1.32-1.37 (t, 3H, $OCH_2\underline{CH_3}$), 4.41-4.43 (q, 2H, $O\underline{CH_2}CH_3$) and 9.10 (br, 2H, NH_2). (See experimental section). **(Scheme 4)**

Compound **14** underwent a variety of interesting reactions resulting in a new number of fused heterocyclic derivatives. Thus compound **14** reacted with acetylacetone to yield 2,4,8-trimethyl-10-phenyl-9(4-methylphenylthio)pyrido [3′,2:4,5] pyrazolo [2,3-b] pyrimidine (**22)**. Its ^{1}H-NMR spectrum revealed signals of additional two CH_3 groups at δ =2.39 and 2.85 ppm. On the other hand, compound **14** reacted with chloroacetonitrile gave 2-amino-7-methyl-9-phenyl-8(4-methylphenylthio)imidazo[1′,2′:1,5] pyrazolo[3,4-b]pyridine **(24),** this compound formed *via* the intermediate of the non-isolable **23.** Compound **14** was established on the basis of its elemental analysis and spectral data. For example, IR spectrum showed NH_2 (3289, 3462) and C=N (1641) and ^{1}H-NMR revealed signals at δ =3.56 (s, 2H, CH_2, $C_{(3)}$-H) and 4.14 (s, 2H, NH_2). Furthermore, compound **14** reacted with chloroacetamide to afford the final isolable **24** through the intermediate **25**, **26**, **and 27. (Scheme 5)**



Scheme 5

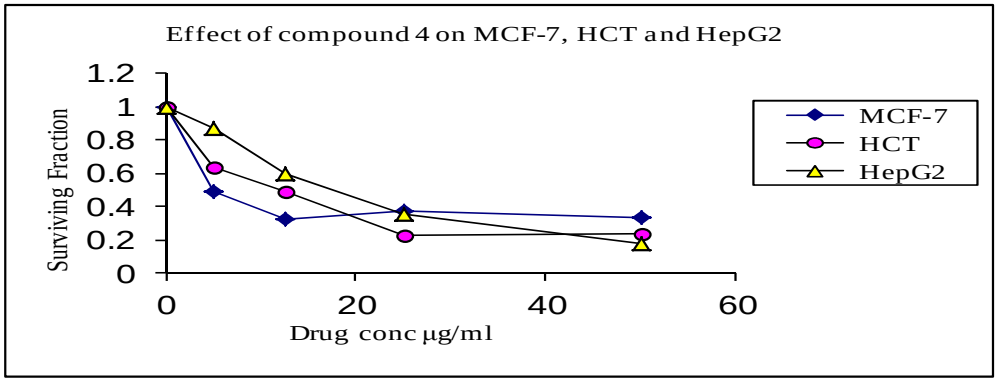
Biological Screening

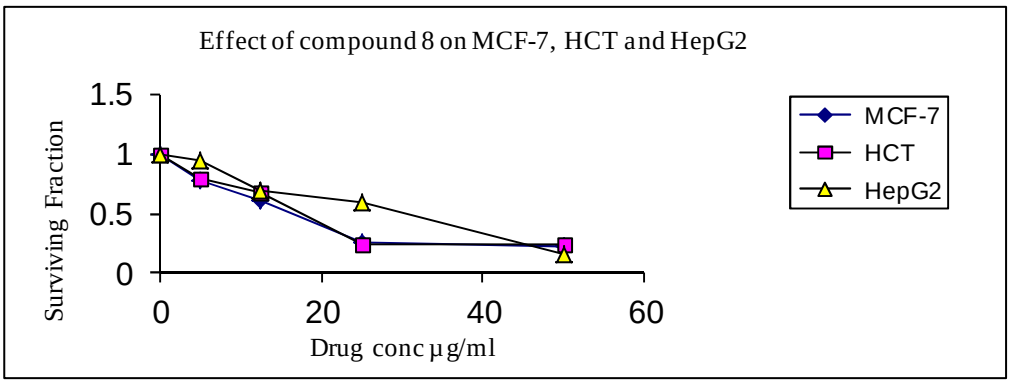
Cytotoxicity against different human cancer cell lines in vitro

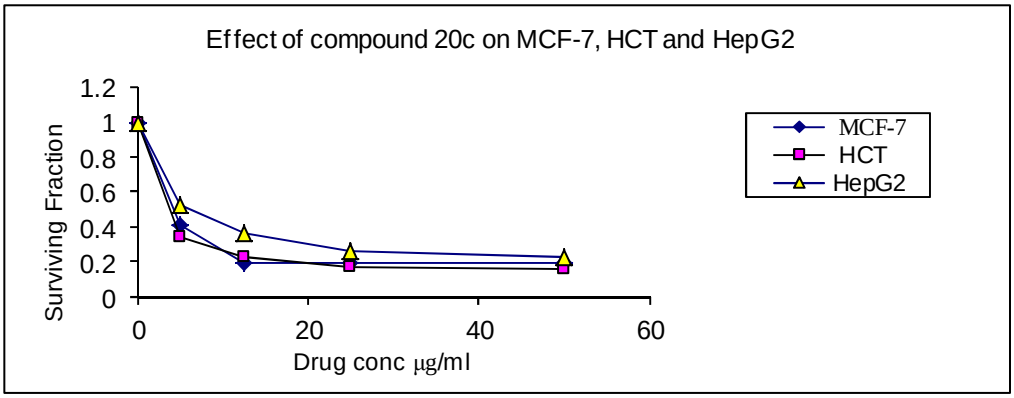
For evolution of anti-tumor cytotoxicity of the synthesized compounds (**4**, **8**, **20C**, **22** and **24**), three different human cancer cell lines were used HepG2 (liver carcinoma cell line), MCF-7 (breast carcinoma cell line) and HCT (colon carcinoma cell line). Cytotoxicity of pyridopyrazolotriazine **20C**, pyridopyrazolopyrimidine **22** pyridopyrazoloimidazobe **24** and pyridothienopyrimidinone **4** and **8** against above human cancer cell lines is shown in fig1. The IC_{50} values are represented in table 1. All The tested substances showed potent anti-tumor toxicity against MCF7 except compound **22** more potent against HCT. The survival fraction was gradually decreased as the concentration of these compounds was increased. Compound 20 c is consider the most potent against three cell lines especially against HCT with the IC_{50} equal to 3.8µg/ml. and compound **8** is the lowest compound against three cell line especially against HepG2 with the IC_{50} equal to 30.4µg/ml.

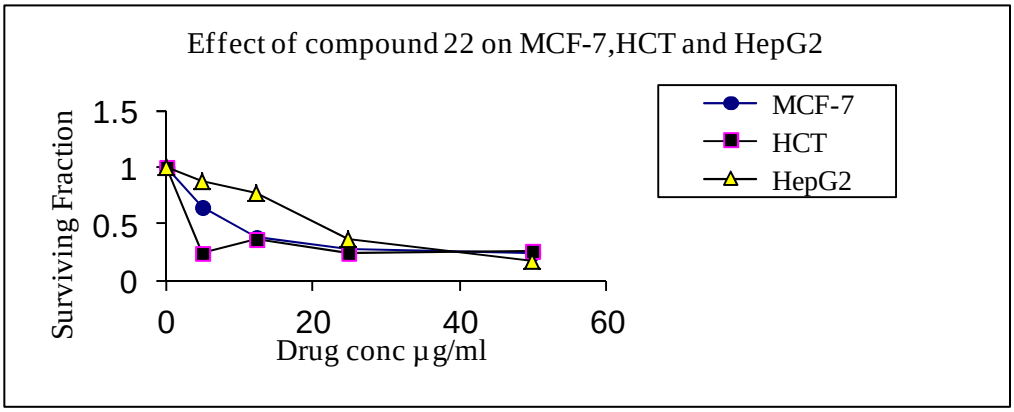
Over all **20c** was more effective in causing anti-tumor cytotoxicity against HCT, MCF-7 and HepG2 cell lines than other compounds. These results are agreement with several publications dealing with other triazine derivatives. It was reported that 1,2,4-triazine compounds have been reported to possess biological activities as anti-AIDS [21] and anticancer [31]. It was reported that 1,3,5-triazine scaffolds with potent activity against Mycobacterium tuberculosis H37Rv [32].

Fig 1. Anti-tumor cytotoxicity of different concentration (IC_{50} µg/ml) of (20c, 24, 22, 4 and 8) against different human cancer lines in vitro









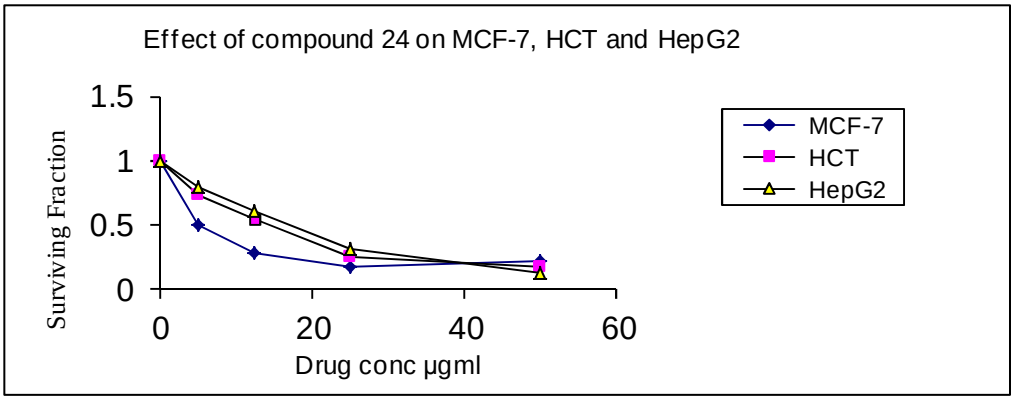


TABLE 1: Effect of the tested compounds on human breast carcinoma cell lines (MCF-7), human liver carcinoma cell lines (HepG2) and human colon carcinoma cell lines (HCT)

IC50[a] µg/ml Tumor cell growth inhibition (%)			
Compounds	MCF-7	HepG2	HCT
4	**4.96**	**17.3**	**11.8**
8	**16.2**	**30.4**	**17.6**
20c	**4.19**	**6.33**	**3.8**
22	**9.22**	**21**	**4.35**
24	**4.9**	**16.8**	**14.1**

[a] *The concentration required for 50% inhibition of cell growth*

CONCLUSION

We have successfully synthesized a series of novel pyrazolo[3,4-b]pyridine and thieno[2,3-*b*]pyridine fused with nitrogen heterocycles such as triazine, pyrimidine, pyrazole and imidazole moiety. The antitumor activity data of the prepared compounds showed that fused heteropolycyclic rings showed good antitumor activity against different human cell lines HepG2, MCF7 and HCT. The best results were obtained by pyridopyrazolotriazine **20c** was the most potent compound in this screening with the IC_{50} equal to 3.8µg/ml.

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