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Utility of Activated Nitriles in the Synthesis of some New Pyridine and Fused Pyridine Derivatives with Anticancer Activity

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ABSTRACT

Objective: This study aimed synthesis of pyridine and fused pyridine derivatives based on the importance of these heterocycles as anticancer. **Method:** Novel pyridine and fused pyridine derivatives **3-35** were synthesized through different chemical reactions. **Results:** Structures of these compounds were confirmed by spectral and elemental analyses. The obtained compounds were evaluated for their in vitro antitumor activity against liver HepG2 and breast MCF-7 cancer cell lines compared to the reference drug (5-fluorouracil). **Conclusion:** Compounds 4, 12, 13, 19, 21, 28 and 29 were found to be the most active against both cell lines exhibiting IC_{50} values ranging from 3.05-11.50 μg/mL and 2.87-6.23 μg/mL against HepG-2 and MCF-7 cell lines; respectively

Keywords: Pyridine; Anticancer; HepG2, MCF-7

INTRODUCTION

Pyridine nuclei are considered as important nitrogen containing heterocyclic systems owing to their several biological activities [1,2]. Among the important biological activities are anticancer [3,4], treatment of Alzheimer's disease [5,6], antibacterial [7], antitubercular [8], antifungal [9], anti-inflammatory [10] activities and the treatment of many cardiovascular diseases such as angina and hypertension[11]. Moreover, pyridine derivatives were found to inhibit the growth of human MCF-7 and HepG-2 cancer cells by exhibiting G2/M phase arrest through a p53-p21-mediated pathway and apoptosis through JNK upregulation [12]. However, numerous cyanopyridines carring lipophilic moieties can inhibit survivin, that is considered as an inhibitor of apoptosis (IAP) family [13]. Also, several 2-oxo-1,2-dihydropyridine-3-carbonitrile derivatives were found to inhibit the oncogenic serine/threonine kinase PIM-1, that included in cancer cell survival, differentiation, and proliferation [14,15]. a series of 1–(2-methyl-6-(4-methoxy/3,4-dimethoxyphenyl)-pyridin-3-yl)-3-phenyl-ureas was developed as promising anti-proliferative agents against breast cancer cell line (MCF-7) [16].

It was reported that hetero ring fused pyridine derivatives were found to exhibit significant anticancer activity against four human cancer cell lines such as
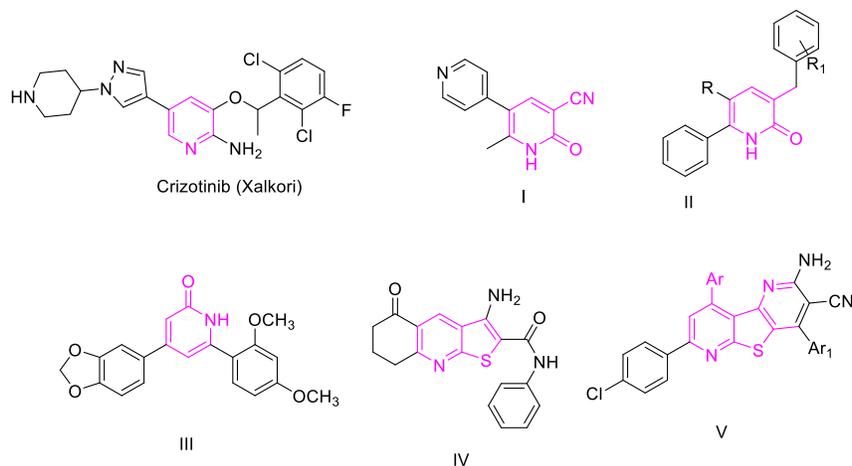
Figure 1. Biologically active pyridine containing anticancer agents.

HeLa (cervical cancer, CCL-2), COLO-205 (colon cancer, CCL-222), HepG2 (liver cancer, HB-8065) and MCF7 (breast cancer, HTB-22) [17,18]. A class of thieno[2,3-b]pyridine compounds have a potent anticancer activity against a variety of tumor cell lines [19,20] such as breast cancer cell line MDA-MB-231[21] and MDA-MB-435 melanoma cell line[22]. While, [1,6]naphthyridines derivatives were found to have anticancer activity against melanoma cell line (MDA-MB-435) [22]. Therefore, our aim was to synthesize and evaluate the anticancer activity of various fused and substituted pyridine analogues.

MATERIAL AND METHODS

Chemistry

All melting points were taken on Electro thermal LA 9000 SERIS, Digital Melting Point Apparatus and were uncorrected. IR Spectra were determined using KBr disk technique on Nikolet IR 200 FT IR Spectrophotometer at Pharmaceutical Analytical Unit, Faculty of Pharmacy, Cairo University, and values are represented in cm⁻¹. The ¹H NMR Spectra was recorded in Varian Gemini EM-300 MHz, NMR Spectrometer at laboratories of the nuclear magnetic resonance, Chemical Warfare Department, Ministry of Defense, DMSO-d_6 was used as a solvent and Chemical shifts were measured in δ ppm, relative to TMS as internal standard. Mass Spectra were recorded at 70 ev on DI-50 unit of Schimadzu GC/MS-QP5050A Spectrometer at Regional Center for Mycology and Biotechnology, Al-Azhar University. Microanalyses were carried out at Regional Center for Mycology and Biotechnology, Al-Azhar University.

Cyanothioacetamide **1** was prepared according to the reported procedure [23,24].

Ethyl 5-cyano-6-mercapto-2-oxo-1,2-dihydropyridine-3-caroxylate; 2

To a solution of sodium metal (0.02 g, 1 mmol) in an absolute ethanol (20 mL), cyanothioacetamide **1** (1 g, 10 mmol) was added. The reaction mixture was stirred for 5 min. during which a solution was formed. After that diethyl ethoxymethylidenmalonate (2.16 g, 2.02 mL, 10 mmol) was added to the reaction mixture and stirring was continued for another 10 min. A precipitate was formed and the mixture was left for 2 h. The precipitate was filtered off, washed with ethanol and hexane and crystallized from DMF/ethanol. Orange powder, 82 % yield; m.p.; 303-305 ºC as reported [25].

Ethyl 2-acetyl-3-amino-6-oxo-6,7-dihydrothieno[2,3-b]pyridine-5-carboxylate; 3

An equimolar mixture of compound **2** (0.45 g, 2 mmol) and chloroacetone (0.18 g, 0.16 mL, 2 mmol) was fused for 20 h. The reaction mixture was allowed to cool then triturated with ethanol. The obtained solid was filtered off and washed with various solvents to give compound **3**.

Black powder, 38 % yield; m.p.; >360 °C, **IR** (KBr, cm⁻¹): 3421, 3414 (br. OH tautomer); 3387, 3292 (NH₂, NH); 3049 (C–H aromatic); 2924, 2854 (C-H aliphatic); 1720, 1710, 1701 (C=O); 1591 (C=N); 1560 (C=C); 1255, 1090 (C–S–C); 1236, 1090 (C–O–C). **¹H-NMR** (DMSO–d₆, δ ppm): 1.08-1.19 (m, 3H, –OCH₂CH₃); 2.55 (s, 3H, CH₃CO); 3.90-4.05 (m, 2H, –OCH₂CH₃); 6.38 (s, 2H, NH₂, D₂O exchangeable); 7.12 (s, 1H, NH, D₂O exchangeable); 7.95 (s, 1H, pyridine–C₄–H). **MS** m/z (relative intensity %): 280 (M⁺•, 2.53). **Anal. Form:** $C_{12}H_{12}N_2O_4S$ (280). **Calcd. (%):** C, 51.42; H, 4.32; N, 9.99; S, 11.44. **Found (%):** C, 51.69; H, 4.37; N, 10.12; S, 11.59.

3-Amino-5-(ethoxycarbonyl)-6-oxo-6,7-dihydrothieno[2,3-b]pyridine-2-carboxylic acid; 4

A mixture of compound **2** (0.45 g, 2 mmol) and chloroacetic acid (0.19 g, 2 mmol) was refluxed for 10 h in a mixture of acetic anhydride and acetic acid (1:2) in presence of anhydrous sodium acetate (0.16 g, 2 mmol). The reaction mixture was allowed to cool then poured onto crushed ice and the obtained solid was filtered, washed with water and crystallized from ethanol to yield compound **4**.

Dark brown powder, 62% yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3446, 3421 (br. OH); 3275, 3228 (NH$_2$, NH); 3057 (C–H aromatic); 2926, 2840 (C–H aliphatic); 1732, 1699, 1683 (C=O); 1570 (C=N); 1558 (C=C); 1242, 1091 (C–S–C & C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.20-1.25 (m, 3H, –OCH$_2$CH$_3$); 4.00-4.12 (m, 2H, –OCH$_2$CH$_3$); 6.13 (s, 2H, NH$_2$, D$_2$O exchangeable); 7.52 (s, 1H, pyridine–C$_4$–H); 8.15 (s, 1H, NH, D$_2$O exchangeable); 11.60 (s, 1H, OH, D$_2$O exchangeable). **MS** m/z (relative intensity %): 283 (M$^{+\bullet}$+1, 3.10); 282 (M$^{+\bullet}$, 6.45); 281(M$^{+\bullet}$-1, 3.76); 280 (M$^{+\bullet}$-2, 1.60). **Anal. Form:** C$_{11}$H$_{10}$N$_2$O$_5$S (282). **Calcd. (%):** C, 46.81; H, 3.57; N, 9.92; S, 11.36. **Found (%):** C, 47.02; H, 3.61; N, 10.04; S, 11.12.

Ethyl 5-cyano-6-(2-ethoxy-2-oxoethylthio)-2-oxo-1,2-dihydropyridine -3-carboxylate; 5 and Diethyl 3-amino-6-oxo-6,7-dihydrothieno[2,3-b]pyridine-2,5-dicarboxylate; 6

Compound **2** (0.45 g, 2 mmol) was suspended in sodium ethoxide solution [prepared by dissolving sodium (0.05 g, 2 mmol) in 10 mL absolute ethanol]and then ethyl chloroacetate (0.24 g, 0.21 mL, 2 mmol) was added and heated under reflux for 12 h. The formed solid was filtered, washed with water several times and crystallized from ethanol to give compound **5,** while the alcohol insoluble part was found to be compound **6**.

Ethyl 5-cyano-6-(2-ethoxy-2-oxoethylthio)-2-oxo-1,2-dihydro pyridine-3-carboxylate; 5

Orange powder, 45 % yield; m.p.; 120-122°C, **IR** (KBr, cm^{-1}): 3429, 3323 (NH); 3049 (C–H aromatic); 2927, 2852 (C–H aliphatic); 2216 (C≡N); 1730, 1710, 1701 (C=O); 1618 (C=N); 1580 (C=C); 1278, 1091 (C–S–C); 1278, 1064 (C–O–C). **^{1}H-NMR** (DMSO–d$_6$, δ ppm): 1.23-1.54 (m, 6 H, two –OCH$_2$CH$_3$); 4.25 (q, 2H, *J* = 7.2 Hz, thiophene-C$_2$-OCH$_2$CH$_3$); 4.41 (q, 2H, *J* = 7.2 Hz, pyridine-C$_3$ –OCH$_2$CH$_3$); 4.70 (s, 1H, SCH$_2$ tautomer); 7.51 (s, 1/2 H, SCH=C–OH tautomer, D$_2$O exchangeable); 7.63 (s, 1/2 H, NH tautomer, D$_2$O exchangeable); 8.32 (s, 1/2 H, SCH=C– tautomer); 8.37 (s, 1H, pyridine–C$_4$–H); 12.48 (s, 1/2 H, pyridine–C$_2$–OH tautomer, D$_2$O exchangeable). **MS** m/z (relative intensity %): 311 (M$^{+\bullet}$+1, 2.41); 310 (M$^{+\bullet}$, 5.21); 309 (M$^{+\bullet}$-1, 33.96). **Anal. Form:** C$_{13}$H$_{14}$N$_2$O$_5$S (310). **Calcd. (%):**

C, 50.31; H, 4.55; N, 9.03; S, 10.33. **Found (%):** C, 50.43; H, 4.59; N, 9.12; S, 10.46.

Diethyl 3-amino-6-oxo-6,7-dihydrothieno[2,3-b]pyridine-2,5- dicarboxyalte; 6

Pale yellow powder, 38 % yield, m.p; 240-242 °C, **IR** (KBr, cm^{-1}): 3446, 3311, 3201 (NH$_2$, NH); 2980 (C–H aromatic); 2860, 2840 (C–H aliphatic); 1720, 1700, 1670 (C=O); 1608 (C=N); 1550 (C=C); 1253, 1064 (C–S–C); 1253, 1037 (C–O–C). **^{1}H-NMR** (DMSO–d$_6$, δ ppm): 1.32 (t, 3H, *J* = 7.1 Hz, thiophene–C$_2$–OCH$_2$CH$_3$); 1.51 (t, 3H, *J* = 7.1 Hz; pyridine–C$_3$–OCH$_2$CH$_3$); 4.30 (q, 2H, *J* = 7.1Hz, thiophene–C$_2$–OCH$_2$CH$_3$); 4.69 (q, 2H, *J* = 7.1 Hz, pyridine–C$_3$–OCH$_2$CH$_3$); 7.63 (s, 2H, NH$_2$, D$_2$O exchangeable); 8.96 (s, 1H, pyridine–C$_4$–H); 8.97 (s, 1H, NH, D$_2$O exchangeable). **MS** m/z (relative intensity %): 310 (M$^{+\bullet}$, 21.64); 309 (M$^{+\bullet}$-1, 100); 308 (M$^{+\bullet}$-2, 6.08). **Anal. form:** C$_{13}$H$_{14}$N$_2$O$_5$S (310). **Calcd. (%):** C, 50.31; H, 4.55; N, 9.03; S, 10.33. **Found (%):** 50.45; H, 4.61; N, 9.08; S, 10.45.

Ethyl 5-cyano-6-[2-(4-methoxyphenyl)-2-oxoethylthio]-2-oxo-1,2-dihydropyridin-3-carboxylate; 7 and Ethyl 3-amino-2-(4-methoxybenzoyl)-6-oxo-6,7-dihydrothieno[2,3-b] pyridine-5-carboxylate; 8

A mixture of compound **2** (0.45 g, 2 mmol), 4-methoxyphenacyl bromide (0.44 g, 2 mmol) and a catalytic amount of anhydrous potassium carbonate (0.28 g, 2 mmol) in dimethylformamide (10 mL) was refluxed for 10 h. The reaction mixture was poured onto crushed ice and the precipitate formed was filtered, washed with water and crystallized from ethanol to give compound **7,** while the alcohol insoluble part was found to be compound **8**.

Ethyl 5-cyano-6-(2-(4-methoxyphenyl)-2-oxoethylthio)-2-oxo-1,2-dihydropyridine-3-carboxylate; 7

Brown powder, 40 % yield, m.p.; 100-102 °C, **IR** (KBr, cm^{-1}): 3406 (br. OH tautomer); 3385, 3284, 3277 (NH); 3101, 3035 (C–H aromatic); 2922, 2835 (C-H aliphatic); 2220 (C≡N); 1690, 1670 (C=O), 1593 (C=N); 1558 (C=C); 1292, 1080 (C–S–C); 1251, 1060 (C–O–C). **^{1}H-NMR** (DMSO–d$_6$, δ ppm): 1.20-1.24 (m, 3H, –OCH$_2$CH$_3$); 3.71 (s, 3H,–OCH$_3$); 3.82-3.87 (m, 2H, –OCH$_2$CH$_3$); 4.20 (s, 1H, S–CH$_2$–C=O tautomer); 7.06 (d, 2H, *J* = 8.5 Hz, 4–OCH$_3$–C$_6$H$_4$–C$_{3,5}$–H); 7.75 (d, 2H, *J* = 8.5 Hz; 4-OCH$_3$–C$_6$H$_4$–C$_{2,6}$–H); 7.86 (s, 1/2 H,–CH=C–OH tautomer); 7.93 (s, 1/2 H, CH=C–OH tautomer, D$_2$O exchangeable); 8.05 (s, 1H, pyridine–C$_4$–H); 8.40 (s, 1/2 H, NH, D$_2$O exchangeable); 10.80 (s, 1/2 H, pyridine–C$_2$–OH tautomer, D$_2$O exchangeable). **MS** m/z (relative intensity %): 372 (M$^{+\bullet}$, 21.13). **Anal. Form:** C$_{18}$H$_{16}$N$_2$O$_5$S (372). **Calcd. (%):** C, 58.05; H, 4.33; N, 7.52; S, 8.61. **Found (%):** C, 58.23; H, 4.41; N, 7.69; S, 8.78.

Ethyl 3-amino-2-(4-methoxybenzoyl)-6-oxo-6,7-dihydrothieno[2,3-b] pyridine-5-carboxylate; 8

Brown powder, 32% yield, m.p.; 240-242 °C, **IR** (KBr, cm^{-1}): 3388, 3269, 3188 (NH$_2$, NH); 3070, 3061 (C–H aromatic); 2926, 2852 (C–H aliphatic); 1720, 1700, 1680 (C=O); 1593 (C=N); 1535 (C=C), 1288, 1060 (C–S–C); 1251, 1024 (C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.20-1.23 (m, 3H, –OCH$_2$CH$_3$); 3.51 (s, 3H, –OCH$_3$); 3.81-3.86 (m, 2H,–OCH$_2$CH$_3$); 5.21 (s, 2H, NH$_2$, D$_2$O exchangeable); 7.00-7.10 (m, 2H, 4–OCH$_3$–C$_6$H$_4$–C$_{3,5}$–H); 7.80-7.93 (m, 2H, 4-OCH$_3$–C$_6$H$_4$–C$_{2,6}$–H); 8.47 (s, 1H, NH, D$_2$O exchangeable); 8.95 (s, 1H, pyridine–C$_4$–H). **MS** m/z (relative intensity %): 372 (M$^{+•}$, 10.00). **Anal. Form:** C$_{18}$H$_{16}$N$_2$O$_5$S (372). **Calcd. (%):** C; 58.05; H, 4.33; N, 7.52; S, 8.61. **Found (%):** C, 58.17; H, 4.39; N, 7.64; S, 8.79.

Ethyl 3-amino-2-cyano-6-oxo-6,7-dihydrothieno[2,3-b]pyridine-5-carboxylate; 9 and Ethyl 5-cyano-6-(cyanomethylthio)-2-oxo-1,2-dihydropyridine-3-carboxylate; 10

Method A:

To a solution of compound **2** (0.45 g, 2 mmol) in dimethylformamide (10 mL), anhydrous potassium carbonate (0.28 g, 2 mmol) was added. The reaction mixture was stirred at room temperature and chloroacetonitrile (0.15 g, 0.13 mL, 2 mmol) was added dropwise while stirring. Stirring was continued at room temperature for 24 h. The reaction mixture was then poured onto cold water and acidified with diluted hydrochloric acid and the obtained product was filtered, washed with water and crystallized from ethanol giving compound **10**, while the alcohol insoluble part was found to be compound **9.**

Method B for preparing compound 9

An equimolar mixture of compound **2** (0.45 g, 2 mmol), anhydrous potassium carbonate (0.28 g, 2 mmol) and chloroacetonitrile (0.15 g, 0.13 mL, 2 mmol) in dimethylformamide (10 mL) was refluxed for 12 h. The reaction mixture was then diluted with acidified water and the obtained product was filtered, washed with water to give compound **9.**

Ethyl 3-amino-2-cyano-6-oxo-5,7-dihydrothieno[2,3-b]pyridine-5-carboxylate; 9

Dark brown powder, 43 % yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3421, 3408 (br. OH tautomer); 3332, 3319, 3149 (NH$_2$, NH); 3057 (C–H aromatic); 2922, 2852 (C–H, aliphatic); 2200 (C≡N); 1700, 1680 (C=O); 1593 (C=N); 1544 (C=C); 1280, 1060 (C–S–C); 1280, 1020 (C–O–C). **^{1}H-NMR** (DMSO–d$_6$, δ ppm): 1.20-1.23 (m, 3H, –OCH$_2$CH$_3$); 3.10-3.30 (m, 2H, –OCH$_2$CH$_3$, under DMSO); 6.89 (s, 2H, NH$_2$, D$_2$O exchangeable); 7.23 (s, 1H, NH, D$_2$O exchangeable); 7.95 (s, 1H, pyridine–C$_4$–H). **MS** m/z (relative intensity %): 264 (M$^{+•}$+1, 24.16); 263 (M$^{+•}$, 4.83); 261 (M$^{+•}$-2,

2.74). **Anal. Form:** C$_{11}$H$_9$N$_3$O$_3$S (263). **Calcd. (%):** C, 50.18; H, 3.45; N, 15.96; S, 12.18. **Found (%):** C, 50.41; H, 3.43; N, 16.08; S, 12.32.

Ethyl 5-cyano-6-(cyanomethylthio)-2-oxo-1,2-dihydropyridine-3-carboxylate; 10.

Dark brown powder, 40 % yield, m.p.; 180-182 °C, **IR** (KBr, cm^{-1}): 3392, 3342, 3170 (NH); 3049 (C–H aromatic); 2924, 2852 (C–H aliphatic); 2191 (C≡N); 1720, 1700 (C=O); 1595 (C=N); 1560 (C=C); 1296, 1095 (C–S–C); 1259, 1095 (C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.20-1.36 (m, 3H, –OCH$_2$CH$_3$); 3.47 (s, 1H, –S–CH$_2$–CN tautomer); 4.10-4.30 (m, 2H, –O–CH$_2$CH$_3$); 6.34 (s, 1/2 H, S–CH=C=NH tautomer, D$_2$O exchangeable); 7.13 (s, 1/2 H, pyridine–NH, D$_2$O exchangeable); 7.46 (s, 1/2 H, pyridine–C$_2$–OH tautomer, D$_2$O exchangeable); 8.32 (s, 1/2 H, S–CH=C=NH tautomer); 8.62 (s, 1H, pyridine–C$_4$–H). **MS** m/z (relative intensity %): 263 (M$^{+•}$, 1.37). **Anal. Form:** C$_{11}$H$_9$N$_3$O$_3$S (263). **Calcd.(%):** C, 50.18; H, 3.45, N, 15.96; S, 12.18; **Found (%):** C, 50.34; H, 3.49; N, 16.04; S, 12.30.

Ethyl 3-amino-2-carbamoyl-6-oxo-6,7-dihydrothieno[2,3-b] pyridine-5-carboxylate; 11

A mixture of 5-cyano-6-mercaptopyridine **2** (0.45 g, 2 mmol), chloroacetamide (0.19 g, 2 mmol) and anhydrous potassium carbonate (0.28 g, 2 mmol) in dimethylformamide (5 mL) was refluxed for 20 h. The reaction mixture was diluted with water and the obtained solid was filtered, washed with water and crystallized from DMF/H$_2$O to give compound **11**.

Dark brown powder, 52 % yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3392 (br. OH tautomer); 3336, 3313, 3196 (NH$_2$, NH), 3064 (C–H aromatic); 2924, 2852 (C–H aliphatic); 1710, 1690, 1660 (C=O); 1604 (C=N); 1541 (C=C); 1286, 1060 (C-S-C); 1271, 1030 (C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.01-1.18 (m, 3H, –OCH$_2$CH$_3$); 4.00-4.03 (m, 2H, –OCH$_2$CH$_3$); 6.75 (s, 2H, thienopyridine–C$_2$–NH$_2$, D$_2$O exchangeable); 7.44 (s, 2H, thienopyridine–C$_3$–NH$_2$, D$_2$O exchangeable); 7.89 (s, 1H, pyridine–C$_4$–H); 8.15 (s, 1H, NH, D$_2$O exchangeable). **MS** m/z (relative intensity %): 281 (M$^{+•}$, 1.87); 279 (M$^{+•}$-2, 0.57). **Anal. Form:** C$_{11}$H$_{11}$N$_3$O$_4$S (281). **Calcd.(%):** C, 46.97; H, 3.94; N, 14.94; S, 11.40; **Found (%):** C, 47.12; H, 3.98; N, 15.08; S, 11.51.

Ethyl 10-amino-2-oxo-1H-6,7,8,9-tetrahydropyrido[3',2':4,5]thieno [3,2-b]quinoline-3-carboxylate;12 and Ethyl 4,7-dioxo-6H-2,2-hexamethylene-1,2,3,4-tetrahydropyrido [3',2' : 4, 5]thieno[3,2-d]pyrimidine-8-carboxylate; 13

An equimolar mixture of compound **9** (0.53 g, 2 mmol) or **11** (0.56 g, 2 mmol) and cyclohexanone (0.2 g, mL, 2 mmol) in dimethylformamide (5 mL) containing

a catalytic amount of zinc chloride (0.27 g, 2 mmol) was refluxed for 20 h. The reaction mixture was allowed to cool and the solid product obtained was filtered off and washed with water to yield compound **12**. The filtrate was poured onto crushed ice and the obtained solid was collected to give compound **13.**

Ethyl 10-amino-2-oxo-1*H*-6,7,8,9-tetrahydropyrido [3',2':4,5]thieno [3,2-b]quinolone; 12

Dark brown powder, DMF/H$_2$O, 53 % yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3406 (br. OH tautomer); 3352, 3307, 3265 (NH$_2$, NH); 3074 (C-H aromatic); 2933 (C-H aliphatic); 1700, 1660 (C=O); 1589 (C=N); 1558 (C=C); 1270, 1064 (C–S–C & C-O-C).**^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.20-1.26 (m, 3H, –O–CH$_2$CH$_3$); 1.80-1.85 (m, 4H, tetrahydroquinoline–C$_{7,8}$–CH$_2$); 3.11-3.19 (m, 2H, tetrahydroquinoline– C$_9$–CH$_2$); 3.40-3.50 (m, 2H, tetrahydroquinoline–C$_6$–CH$_2$); 3.55-3.61 (m, 2H, –O–CH$_2$CH$_3$); 5.84 (s, 2H, NH$_2$, D$_2$O exchangeable); 7.19 (s, 1H, NH, D$_2$O exchangeable); 8.08 (s, 1H, thienopyridine–C$_4$–H). **MS** m/z (relative intensity %): 345 (M$^{+\bullet}$+2, 2.23); 343 (M$^{+\bullet}$, 3.76). **Anal. Form:** C$_{17}$H$_{17}$N$_3$O$_3$S (343). **Calcd. (%):** C, 59.46; H, 4.99; N, 12.24; S, 9.34. **Found (%):** C, 59.64; H, 5.08; N, 12.32; S, 9.49.

Ethyl 4,7-dioxo-6*H*-2,2-hexamethylene-1,2,3,4-tetrahydropyrido [3',2': 4,5]thieno[3,2-d]pyrimidine-8-carboxylate; 13

Buff powder, DMF/EtOH, 32 % yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3448 (br. OH tautomer); 3207, 3192 (NH); 3026 (C-H aromatic); 2924, 2854 (C–H aliphatic); 1700, 1680, 1670 (C=O); 1587 (C=N); 1255, 1103 (C–S–C); 1255, 1043 (C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.19-1.24 (m, 3H, –O–CH$_2$CH$_3$); 1.70-1.83 (m, 6H, cyclohexyl-C$_{3,4,5}$–CH$_2$); 2.20-2.27 (m, 4H, cyclohexyl-C$_{2,6}$–CH$_2$); 3.52-3.61 (m, 2H, –O–CH$_2$CH$_3$); 7.95 (s, 1H, pyrimidine–N$_1$–H, D$_2$O exchangeable); 8.22 (s, 1H, thienopyridine–C$_9$–H); 12.19 (s, 2H, thienopyridine–N$_6$–H, & pyrimidine–N$_3$–H, D$_2$O exchangeable). **MS** m/z (relative intensity %): 361 (M$^{+\bullet}$, 2.80). **Anal form:** C$_{17}$H$_{19}$N$_3$O$_4$S (361). **Calcd. (%):** C, 56.50; H, 5.30; N, 11.63; S, 8.87. **Found (%):** C, 56.67; H, 5.37, N, 11.80; S, 9.04.

Ethyl 2-acetyl-3-imino-2-methyl-6-oxo-2,3,6,7-tetrahydrothieno[2,3-b]pyridine-5-carboxylate; 14, Ethyl 3'-imino-2,6'-dioxo-6',7'-dihydro-3'*H*-spiro[cyclopentane-1,2'-thieno[2,3-b]pyridine-5'-carboxylate; 15 and Ethyl 2,2-diacetyl-3-imino-6-oxo-2,3,6,7-tetrahydrothieno[2,3-b] pyridine-5-carboxylate; 16
General procedure

A mixture of 5-cyano-6-mercaptopyridine **2** (0.45 g, 2 mmol) and the appropriate ketone (2 mmole) namely; ethylmethyl ketone, cyclopentanone and acetylacetone; respectively was refluxed in acetic acid (5 mL) containing a few drops of conc. H$_2$SO$_4$ (3-5 drops) for 2 h, then acetic anhydride (2 mL) was added to the reaction mixture and refluxing was continued for another 2 h. The reaction mixture was cooled and the obtained products were filtered and crystallized from the appropriate solvent to yield compounds **14, 15** and **16**; respectively.

Ethyl 2-acetyl-3-imino-2-methyl-6-oxo-2,3,6,7-tetrahydrothieno[2,3-b]pyridine-5-carboxylate; 14

Dark brown powder, boiled with ethanol, methanol, ethylacetate and benzene, 62 % yield, m.p.; >360 °C, **IR** (KBr, cm^{-1}): 3433, 3423 (br. OH tautomer); 3392, 3232 (NH); 3034 (C-H aromatic); 2926 (C–H aliphatic); 1720, 1701, 1690 (C=O); 1630 (C=N); 1541 (C=C); 1217, 1035 (C–S–C & C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.85-1.90 (m, 3H, –O–CH$_2$CH$_3$); 2.71 (s, 3H, thiophene–C$_2$–CH$_3$); 2.87 (s, 3H, O=C–CH$_3$); 3.38-3.57 (m, 2H, –O–CH$_2$CH$_3$); 6.90 (s, 1H, C=NH, D$_2$O exchangeable); 7.07 (s, 1/2 H, NH, D$_2$O exchangeable); 7.24 (s, 1/2 H, OH tautomer, D$_2$O exchangeable); 7.95 (s, 1H, pyridine–C$_4$–H). **MS** m/z (relative intensity %): 295 (M$^{+\bullet}$+1, 3.59); 294 (M$^{+\bullet}$, 4.21).**Anal. Form:** C$_{13}$H$_{14}$N$_2$O$_4$S (294). **Calcd. (%):** C, 53.05; H, 4.79; N, 9.52; S, 10.89. **Found (%):** C, 53.21; H, 4.85; N, 9.67; S, 11.01.

Ethyl 3'-imino-2,6'-dioxo-6',7'-dihydro-3'*H*-spiro[cyclopentane-1,2'-thieno[2,3-b]pyridine]-5'-carboxylate; 15

Dark brown powder, EtOH/DMF, 83% yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3356 (br. OH tautomer); 3275, 3194 (NH); 3059 (C–H aromatic); 2924, 2854 (C–H aliphatic); 1680, 1654 (C=O); 1597 (C=N); 1558 (C=C); 1265, 1091 (C–S–C & C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.19 (s, 3H, –O–CH$_2$CH$_3$); 2.73 (s, 2H, cyclopentanone–C$_4$–CH$_2$); 2.89 (s, 2H, cyclopentonone–C$_5$–CH$_2$); 3.40 (s, 2H, cyclopentanone–C$_3$–CH$_2$); 4.05 (s, 2H, –O–CH$_2$CH$_3$); 6.89 (s, 1H, C=NH, D$_2$O exchangeable); 7.05 (s, 1/2H, NH, D$_2$O exchangeable); 7.23 (s,1/2 H, OH tautomer, D$_2$O exchangeable); 7.95 (s, 1H, pyridine–C$_4$–H). **MS** m/z (relative intensity %): 308 (M$^{+\bullet}$+2, 2.72); 306 (M$^{+\bullet}$, 1.20). **Anal. Form:** C$_{14}$H$_{14}$N$_2$O$_4$S (306). **Calcd. (%):** C, 54.89; H, 4.61; N, 9.14; S, 10.47. **Found (%):** C, 55.03; H, 4.67; N, 9.21; S, 10.59.

Ethyl 2,2-diacetyl-3-imino-6-oxo-2,3,6,7-tetrahydrothieno[2,3-b] pyridine-5-carboxylate; 16

Dark brown powder, boild with ethanol, methanol, ethylacetate and benzene, 65 % yield, m.p.; >360 °C, **IR** (KBr, cm^{-1}): 3446, 3400 (br. OH tautomer); 3387, 3369 (NH); 3049 (C-H aromatic); 2924 (C–H aliphatic); 1730, 1716, 1697, 1683 (C=O); 1620 (C=N); 1558 (C=C); 1313, 1033 (C–S–C); 1219, 1033 (C–O–C).

^{1}H-NMR (DMSO-d_6, δ ppm): 1.90 (s, 3H,–O–CH$_2$<u>CH$_3$</u>); 2.89 (s, 6H, two <u>CH$_3$</u>C=O); 3.40 (s, 2H, –O–<u>CH$_2$</u>CH$_3$); 6.90 (s, 1H, C=NH , D$_2$O exchangeable); 7.06 (s, 1/2 H, NH, D$_2$O exchangeable); 7.24 (s, 1/2 H, OH tautomer, D$_2$O exchangeable); 7.95 (s, 1H, pyridine– C$_4$–H). **MS** m/z (relative intensity %): 322 (M$^{+•}$, 0.97). **Anal. form:** C$_{14}$H$_{14}$N$_2$O$_5$S (322). **Calcd. (%):** C, 52.17; H, 4.38; N, 8.69; S, 9.95. **Found (%):** C, 52.32; H, 4.42; N, 8.81; S, 10.03.

Ethyl 5-cyano-6-(1-ethoxy-1,3-dioxobutan-2-ylthio)-2-oxo-1,2-dihydropyridine-3-carboxylate; 17, and Diethyl 2-acetyl-3-imino-6-oxo-2,3,6,7-tetrahydro thieno[2,3-b] pyridine-2,5-dicarboxylate; 18

To a stirred solution of 5-cyano-6-mercaptopyridine **2** (0.45 g, 2 mmol) in ethanol/dimethylformamide (4:1) (10 mL) aqueous potassium hydroxide solution (0.11 g, 2 mmol) (2 mL) was added. To the resulting solution ethyl 2-chloro-3-oxobutanoate (0.33 g, 0.28 mL, 2 mmol) was added and the reaction mixture was stirred for 24 h, then diluted with water. The precipitated solid was filtered off, washed with water, dried and crystallized from dioxane/ethanol to give compound **17** while the insoluble part was found to be compound **18.**

Ethyl 5-cyano-6-(1-ethoxy-1,3-dioxobutan-2-ylthio)-2-oxo-1,2-dihydropyridine-3-carboxylate; 17

Buff powder, 45 % yield, m.p.; 95-97 °C, **IR** (KBr, cm^{-1}): 3410 (OH tautomer); 3317, 3221 (NH), 3097, 2970 (C–H aromatic); 2924, 2850 (C–H aliphatic); 2206 (C≡N); 1728, 1680 (C=O); 1631 (C=N); 1539, 1512 (C=C); 1300, 1026 (C–S–C); 1261, 1026 (C–O–C). **^{1}H-NMR** (DMSO-d_6, δ ppm): 1.24-1.34 (m, 6H, two –O–CH$_2$<u>CH$_3$</u>); 2.71 (s, 3H, <u>CH$_3$</u>–C=O); 4.19 (q, 2H, *J*= 7.2 Hz, 3-oxobutanoate–C$_1$–O–<u>CH$_2$</u>CH$_3$); 4.29 (q, 2H, *J*=7.1 Hz, pyridine–C$_3$–COO–<u>CH$_2$</u>CH$_3$); 5.79 (s, 1H, 3-oxobutanoate–C$_2$–H); 7.29 (s, 1/2 H, NH, D$_2$O exchangeable); 8.15 (s, 1H, pyridine–C$_4$–H); 12.10 (s, 1/2 H, OH tautomer, D$_2$O exchangeable). **MS** m/z (relative intensity %): 352 (M$^{+•}$, 0.71); 350 (M$^{+•}$-2, 3.04). **Anal. Form:** C$_{15}$H$_{16}$N$_2$O$_6$S (352). **Calcd. (%):** C, 51.13; H, 4.58; N, 7.95; S, 9.10. **Found (%):** C, 51.40; H, 4.66; N, 8.05; S, 9.17.

Diethyl 2-acetyl-3-imino-6-oxo-2,3,6,7-tetrahydro thieno[2,3-b] pyridine-2,5-dicarboxylate; 18

Brown powder, 40 % yield, m.p.; > 360°C, **IR** (KBr, cm^{-1}): 3431 (OH tautomer); 3261, 3186 (NH); 3000 (C-H aromatic); 2920 (C–H aliphatic); 1730, 1670 (C=O); 1616 (C=N); 1550 (C=C); 1301, 1093 (C–S–C); 1265, 1066 (C–O–C). **^{1}H-NMR** (DMSO-d_6, δ ppm): 1.23-1.33 (m, 6H, two –O–CH$_2$<u>CH$_3$</u>); 2.71 (s, 3H, <u>CH$_3$</u>C=O); 4.17 (q, 2H, *J*=6.7Hz, 3-oxobutanoate–C$_1$–O–<u>CH$_2$</u>CH$_3$); 4.28 (q, 2H, *J*=6.7Hz, pyridine–C$_3$–COO–<u>CH$_2$</u>CH$_3$); 5.80 (s, 1H, C=NH, D$_2$O exchangeable); 7.29

(s, 1/2 H, NH, D$_2$O exchangeable); 8.18 (s, 1H, pyridine–C$_4$–H); 12.18 (s, 1/2 H, OH tautomer, D$_2$O exchangeable). **MS** m/z (relative intensity %): 352 (M$^{+•}$, 2.99). **Anal. Form:** C$_{15}$H$_{16}$N$_2$O$_6$S (352). **Calcd. (%):** C, 51.13; H, 4.58; N, 7.95; S, 9.10. **Found (%):** C, 51.29; H, 4.64; N, 8.07; S, 9.15.

Ethyl 6-(2-chloro-2-oxoacetylthio)-5-cyano-2-oxo-1,2-dihydro pyridine-3-carboxylate; 19 and
Ethyl 8-cyano-2,3,5-trioxo-3,5-dihydro-2*H*-thiazolo[3,2-a]pyridine-6-carboxylate; 20

A mixture of 5-cyano-6-mercaptopyridine **2** (0.45 g, 2 mmol) and oxalylchloride (0.25 g, 0.17 mL, 2 mmol) was fused at 160-170 °C for 30 min. The reaction mixture was then triturated with ethanol. The obtained precipitate was filtered, washed with diethyl ether and crystallized from ethanol to give compound **19** while the alcoholic insoluble part was found to be compound **20.**

Ethyl 6-(2-chloro-2-oxoacetylthio)-5-cyano-2-oxo-1,2-dihydropyridine-3-carboxylate; 19

Dark brown powder, 36 % yield, m.p.; 117-119 °C, **IR** (KBr, cm^{-1}): 3425, 3414 (br. OH tautomer); 3238, 3192 (NH); 2993 (C-H aromatic); 2926, 2850 (C–H aliphatic); 2223 (C≡N); 1737, 1697 (C=O); 1602 (C=N); 1550 (C=C); 1286, 1070 (C–S–C); 1249, 1070 (C–O–C). **^{1}H-NMR** (DMSO-d_6, δ ppm): 1.42 (t, 3H, *J* = 3.4 Hz, –OCH$_2$ <u>CH$_3$</u>); 4.55 (q, 2H, *J* = 7.1 Hz, –O<u>CH$_2$</u>CH$_3$); 8.15 (s, 1/2 H, NH, D$_2$O exchangeable); 8.39 (s, 1H, pyridine–C$_4$–H); 12.77 (s, 1/2 H, OH tautomer, D$_2$O exchangeable). **MS** m/z (relative intensity %): 314 (M$^{+•}$, 0.79). **Anal. Form:** C$_{11}$H$_7$ClN$_2$O$_5$S (314). **Calcd.(%):** C, 41.98; H, 2.24; N, 8.90; S, 10.19. **Found (%):** C, 42.13; H, 2.21; N, 8. 98; S, 10.31.

Ethyl 8-cyano-2,3,5-trioxo-3,5-dihydro-2*H*-thiazolo[3,2-a]pyridine-6-carboxylate; 20

Dark brown crystals, 38 % yield, m.p.; >360 °C, **IR** (KBr, cm^{-1}): 3068, 3016 (C–H aromatic); 2924, 2854 (C–H aliphatic); 2214 (C≡N); 1701, 1680, 1670 (C=O); 1622, 1595 (C=N); 1558, 1535 (C=C); 1273, 1060 (C–S–C); 1244, 1060 (C–O–C). **^{1}H-NMR** (DMSO-d_6, δ ppm): 1.21 (t, 3H, *J* = 6Hz, –OCH$_2$<u>CH$_3$</u>); 4.05-4.38 (m, 2H, –O<u>CH$_2$</u>CH$_3$); 8.51 (s, 1H, pyridine–C$_4$–H). **MS** m/z (relative intensity %): 278 (M$^{+•}$, 2.01). **Anal. Form:** C$_{11}$H$_6$N$_2$O$_5$S (278). **Calcd. (%):** C, 47.48; H, 2.17; N, 10.07; S, 11.52. **Found (%):** C, 47.62; H, 2.19; N, 10.23; S, 11.60.

Ethyl 5-cyano-6-(ethoxycarbonylthio)-2-oxo-1,2-dihydropyridine-3-carboxylate; 21

A mixture of 5-cyano-6-mercaptopyridine **2** (0.45 g, 2 mmol) and ethyl chloroformate (0.22 g, 0.19 mL, 2 mmol) containing a catalytic amount of anhydrous potassium carbonate (0.28 g, 2 mmol) in dry dimethylformamide (10 mL) was refluxed for 20 h. The

reaction mixture was poured onto crushed ice, acidified with diluted hydrochloric acid and the obtained produced was filtered, washed with water and crystallized from ethanol to give compound **21**.

Yellow powder, 32 % yield, m.p.; 140-142 °C, **IR** (KBr, cm⁻¹): 3414 (br. OH tautomer); 3228, 3170 (NH); 3057, 3016 (C-H aromatic); 2924, 2852 (C–H aliphatic); 2223 (C≡N); 1720, 1701, 1670 (C=O); 1620, 1589 (C=N); 1550 (C=C); 1253, 1082 (C–S–C); 1232, 1082 (C–O–C). **MS** m/z (relative intensity %): 296 (M⁺•, 2.02); 295 (M⁺•-1, 0.96). **Anal. Form:** $C_{12}H_{12}N_2O_5S$ (296). **Calcd.(%):** C, 48.64; H, 4.08; N, 9.45; S, 10.82. **Found (%):** C, 48.76; H, 4.11; N, 9.61; S, 10.96.

Ethyl 8-cyano-2-(2-methoxy-2-oxoethylidene)-3,5-dioxo-3,5-dihydro-2*H*-thiazolo[3,2-a]pyridine-6-carboxylate; 22

An equimolar mixture of 5-cyano-6-mercaptopyridine **2** (0.45 g, 2 mmol) and dimethyl acetylene dicarboxylate (0.28 g, 0.25 mL, 2 mmol) was fused at 160-170 °C for 8 h. The reaction mixture was left to cool then triturated with absolute ethanol. The obtained product was filtered, washed with diethyl ether, left to dry and crystallized from ethanol to give compound **22.**

Dark brown powder, 80 % yield, m.p.; 224-226 °C, **IR** (KBr, cm⁻¹): 3005 (C-H aromatic); 2954, 2926, 2852 (C–H aliphatic); 2240 (C≡N); 1735, 1716, 1690 (C=O); 1540 (C=C); 1240, 1070 (C–S–C & C–O–C). **¹H-NMR** (DMSO-d₆, δ ppm): 1.16-1.24 (m, 3H, –OCH₂CH₃); 3.44-4.05 (m, 5H, –OCH₂CH₃ & –OCH₃); 5.05 (s, 1H, C=CH-COOCH₃); 7.91 (s, 1H, pyridine–C₄–H). **MS** m/z (relative intensity %): 334 (M⁺•, 0.99). **Anal Form:** $C_{14}H_{10}N_2O_6S$ (334). **Calcd.(%):** C, 50.30; H, 3.02; N, 8.38; S, 9.59. **Found (%):** C, 50.42; H, 3.05; N, 8.47; S, 9.72.

Ethyl 3-acetyl-1-(4-chlorophenyl)-8-cyano-5-oxo-1,5-dihydro[1,2,4] triazolo[4,3-a]pyridine-6-carboxylate; 24

To a solution of compound **2** (0.45 g, 2 mmol) in dimethyl formamide (5 mL), 4-chlorophenyl-2-oxopropane hydrazonoyl chloride [26] **23** (0.46 g, 2 mmol) and a catalytic amount of anhydrous potassium carbonate (0.28 g, 2 mmol) were added. The reaction mixture was refluxed for 12 h, allowed to cool and the precipitated product was filtered off, washed with water, and crystallized from ethanol to give compound **24**.
Brown powder, 43 % yield, m.p.; 240-242 °C, **IR** (KBr, cm⁻¹): 3051 (C-H aromatic); 2924, 2852 (C–H aliphatic); 2200 (C≡N); 1700, 1680, 1670 (C=O); 1610 (C=N); 1560 (C=C); 1288, 1091(C–O–C); 1091 (p-Cl-phenyl); 829 (C–Cl). **¹H-NMR** (DMSO-d₆, δ ppm): 1.12-1.30 (m, 3H, –OCH₂CH₃); 2.89 (s, 3H, CH₃CO–); 4.07-4.19 (m, 2H, –OCH₂CH₃); 7.33-7.51 (m, 2H, 4-Cl-C₆H₄-C₂,₆ –H); 7.95 (s, 1H, pyridine C₄–H); 8.45-8.61 (m, 2H, 4-Cl-

C₆H₄-C₃,₅–H). **MS** m/z (relative intensity %): 384 (M⁺•, 1.66). **Anal. Form:** $C_{18}H_{13}ClN_4O_4$ (384). **Calcd. (%):** C, 56.19; H, 3.41; N, 14.56. **Found (%):** C, 56. 48; H, 3.48; N, 14.72.

Ethyl 5-cyano-6-(methylthio)-2-oxo-1,2-dihydropyridine-3-carboxylate; 25

A mixture of compound **2** (0.45 g, 2 mmol) and potassium hydroxide (0.11 g, 2 mmol) in dimethylformamide (10 mL) was stirred for 2 h at room temperature, then methyl iodide (0.28 g, 0.12 mL, 2 mmol) was added and stirring was continued for another 2 h. The reaction mixture was poured on ice cold water, acidified with diluted hydrochloric acid, filtered, washed with water and crystallized from ethanol to give compound **25**.

Orange powder, 55 % yield, m.p.; 205-207 °C, **IR** (KBr, cm⁻¹): 3404 (br. OH tautomer); 3385, 3365 (NH); 3047 (C-H aromatic); 2926, 2852 (C–H aliphatic); 2227 (C≡N); 1710, 1660 (C=O); 1587 (C=N); 1529 (C=C); 1251, 1070 (C–S–C & C–O–C). **MS** m/z (relative intensity %): 238 (M⁺•, 1.14). **Anal. Form:** $C_{10}H_{10}N_2O_3S$ (238). **Calcd. (%):** C, 50.41; H, 4.23; N, 11.76; S, 13.46. **Found (%):** C, 50.53; H, 4.31; N, 11.89; S, 13.61.

5-Amino-6,8-dihydrodipyrazolo[3,4-b: 4',3'-e]pyridine-3(2*H*)-one; 26

To a suspension of the S-methyl derivative **25** (0.48 g, 2 mmol) in absolute ethanol (10 mL), hydrazine hydrate (0.2 g, 0.19 mL, 4 mmol) was added and the reaction mixture was heated under reflux for 12 h. It was concentrated and the obtained product was filtered, washed with several solvents to yield compound **26**.
Yellow powder, 38 % yield, m.p.; > 360 °C, **IR** (KBr, cm⁻¹): 3300, 3188, 3138 (NH₂, NH); 3000 (C-H aromatic); 2922 (C–H aliphatic); 1690 (C=O); 1624 (C=N); 1590 (C=C). **¹H-NMR** (DMSO-d₆, δ ppm): 5.23 (s, 2H, NH₂, D₂O exchangeable); 6.21 (s, 2H, two pyrazole–NH, D₂O exchangeable); 7.02 (s, 1H, pyridine–NH, D₂O exchangeable); 8.45 (s, 1H, pyridine–C₄–H). **MS** m/z (relative intensity %): 191 (M⁺•+1, 1.97); 190 (M⁺•, 2.46); 189 (M⁺•-1, 5.98); 188 (M⁺•-2, 3.68). **Anal. Form:** $C_7H_6N_6O$ (190). **Calcd. (%):** C, 44.21; H, 3.18; N, 44.19. **Found (%):** C, 44.34; H, 3.25; N, 44.52.

Cinnamoyl chloride; 27

A mixture of cinnamic acid (1.4 g, 10 mmol) and thionyl chloride (1.7 g, 1.1 mL, 15 mmol) was stirred at room temperature for 15 min. Stirring was then completed for additional 2 h at 80 ºC. The obtained solid was collected and dried to yield yellow crystals of cinnamoyl chloride.Yield %; 75 %, m.p.; 33-34ºC (Lit.[27], 30-33 ºC).

Ethyl 6-(cinnamoylthio)-5-cyano-2-oxo-1,2-dihydropyridine-3-carboxylate; 28 ; and Ethyl 9-

cyano-2,6-dioxo-4-phenyl-2,3,4,6-tetrahydropyrido [2,1-b][1,3]thiazine-7-carboxylate; 29

An equimolar mixture of 5-cyano-6-mercaptopyridine **2** (0.45 g, 2 mmol) and cinnamoyl chloride **27** (0.33 g, 2 mmol) was refluxed for 20 h in a mixture of pyridine and benzene (1:1) (10 mL). The reaction mixture was allowed to cool then poured onto crushed ice while scratching then the solid formed was filtered off, dried and crystallized from ethanol to give compound **28**, while the alcoholic insoluble part was found to be compound **29**.

Ethyl 6-(cinnamoylthio)-5-cyano-2-oxo-1,2-dihydropyridine-3-carboxylate; 28

Yellow powder, 39 % yield, m.p.; 134-136 °C, **IR** (KBr, cm^{-1}): 3380, 3340 (NH); 3066, 3026 (C-H aromatic); 2924, 2852 (C–H aliphatic); 2220 (C≡N); 1720, 1700, 1680 (C=O); 1629 (C=N); 1550 (C=C); 1284, 1074 (C–S–C); 1265, 1074 (C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.21 (t, 3H, J = 7.1 Hz, -OCH$_2$CH$_3$); 4.09 (q, 2H, J= 7.1Hz, –OCH$_2$CH$_3$); 6.52 (d, 1H, J=15.9 Hz, =CH–C=O); 7.34-7.52 (m, 3H, C$_6$H$_5$–C$_{3,4,5}$–H); 7.59 (d, 1H, J = 15.9 Hz, =CH– C$_6$H$_5$); 7.62-7.77 (m, 2H, C$_6$H$_5$–C$_{2,6}$–H); 7.81 (s, 1H, pyridine–C$_4$–H); 11.10 (s, 1H, OH tautomer, D$_2$O exchangeable). **MS** m/z (relative intensity %): 354 (M$^{+•}$, 2.32). **Anal. Form:** C$_{18}$H$_{14}$N$_2$O$_4$S (354). **Calcd. (%):** C, 61.01; H, 3.98; N, 7.90; S, 9.05. **Found(%):** C, 61.24, H, 3.96; N, 7. 98; S, 9.17.

Ethyl 9-cyano-2,6-dioxo-4-phenyl-2,3,4,6-tetrahydropyrido[2,1-b] [1,3]thiazine-7-carboxylate; 29

Orange powder, 38 % yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3410 (br. OH tautomer); 3049, 3003 (C–H aromatic); 2922, 2852 (C–H aliphatic); 2210 (C≡N); 1710, 1680 (C=O); 1585 (C=C); 1269, 1091 (C–S–C & C–O–C). ^{1}H-NMR (DMSO–d$_6$, δ ppm): 1.02-1.22 (m, 3H, –OCH$_2$CH$_3$); 3.81-3.85 (m, 2H, –OCH$_2$CH$_3$); 7.36-7.40 (m, 1H, thiazine– C$_4$–H); 7.77 (d, 1H, J=7.5 Hz, thiazine–C$_5$–H); 8.25-8.34 (m, 3H, C$_6$H$_5$– C$_{3,4,5}$–H); 8.49-8.51 (m, 2H, C$_6$H$_5$–C$_{2,6}$–H); 8.59 (s, 1H, pyridine–C$_4$–H); 14.38 (s, 1H, OH, D$_2$O exchangeable). **MS** m/z (relative intensity %): 356 (M$^{+•}$+2, 2.10); 354 (M$^{+•}$, 2.46). **Anal. Form:** C$_{18}$H$_{14}$N$_2$O$_4$S (354). **Calcd. (%):** C, 61.01; H, 3.98; N, 7.90; S, 9.05. **Found (%):** C, 61.18; H, 4.03; N, 7.98; S, 9.14.

2,4-Dimethoxybenzylidene malononitrile; 30

2,4-Dimethoxybenzaldehyde (3.32 g, 20 mmol) was added to a solution of malononitrile (1.32 mL, 20.8 mmol) in ethanol (14 mL) containing a catalytic amount of piperdine (3 drops). The reaction mixture was refluxed for one hour and allowed to cool to room temperature. The obtained solid was filtered off, washed with ethanol and dried to yield yellow crystals. Yield %; 92 %, m.p.; 144-146 °C (Lit. [28], 149-152 °C).

Ethyl 3-cyano-2--(2,4-dimethoxyphenyl)-4-imino-7-oxo-7,8-dihydro-4H-thiopyrano[2,3-b]pyridine-6-carboxylate; 31; and Ethyl 9-cyano-2-(2,4-dimethoxyphenyl)-4-imino-6-oxo-4,6-dihydro pyrido[2,1-b][1,3]thiazine-7-carboxylate; 32

An equimolar mixture of 5-cyano-6-mercaptopyridine **2** (0.45 g, 2 mmol) and 2,4-dimethoxybenzylidenemalononitrile **30** (0.43 g, 2 mmol) was refluxed for 20 h in dimethylformamide (5 mL) containing a catalytic amount of potassium carbonate (0.28 g, 2 mmole). The reaction mixture was allowed to cool then poured onto cold water. The solid formed was filtered off, washed with water then dried and crystallized from ethanol to give compound **31**, while the insoluble part was found to be compound **32**.

Ethyl 3-cyano-2-(2,4-dimethoxyphenyl)-4-imino-7-oxo-7,8-dihydro-4H-thiopyrano[2,3-b]pyridine-6-carboxylate; 31

Brown powder, 30 % yield, m.p.; 80-82 °C, **IR** (KBr, cm^{-1}): 3427, 3412 (br. OH tautomer); 3211, 3190 (NH); 3049, 3007 (C–H aromatic); 2926, 2852 (C–H aliphatic); 2214 (C≡N); 1720, 1650 (C=O); 1606 (C=N); 1544 (C=C); 1253, 1099 (C–S–C); 1253, 1022 (C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.10-1.23 (m, 3H, -OCH$_2$CH$_3$); 3.62-3.86 (m, 2H, -OCH$_2$CH$_3$); 4.03 (s, 6H, two OCH$_3$); 6.50-6.90 (m, 1H, 2,4-(OCH$_3$)$_2$-C$_6$H$_3$-C$_5$– H); 7.22 (s, 1H, 2,4-(OCH$_3$)$_2$-C$_6$H$_3$-C$_6$–H); 7.90 (s, 1H, 2,4-(OCH$_3$)$_2$-C$_6$H$_3$-C$_3$–H); 8.15 (s, 1H, pyridine–NH, D$_2$O exchangeable); 8.41 (s, 1H, pyridine–C$_4$–H); 8.68 (s, 1H, imino-NH, D$_2$O exchangeable). **MS** m/z (relative intensity %): 411 (M$^{+•}$, 0.88). **Anal. Form:** C$_{20}$H$_{17}$N$_3$O$_5$S (411). **Calcd.(%):** C, 58.38; H, 4.16; N, 10.21; S, 7.79. **Found (%):** C, 58.51; H, 4.22; N, 10.34; S, 7.84.

Ethyl 9-cyano-2-(2,4-dimethoxyphenyl)-4-imino-6-oxo-4,6-dihydro pyrido[2,1-b][1,3]thiazine-7-carboxylate; 32

Orange powder, 42 % yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3363, 3188 (NH); 3072, 3014 (C–H aromatic); 2931, 2841(C–H aliphatic); 2214 (C≡N); 1700, 1680 (C=O);1610, 1597 (C=N); 1560, 1541 (C=C); 1253, 1024 (C-S-C & C–O–C). **MS** m/z (relative intensity %): 411 (M$^{+•}$, 0.82). **Anal. Form:** C$_{20}$H$_{17}$N$_3$O$_5$S (411). **Calcd.(%):** C, 58.38; H, 4.16; N, 10.21; S, 7.79. **Found(%):** C, 58.53; H, 4.20; N, 10.32; S, 7.87.

Ethyl 2-chloromethyl-6H, 9H-4,7-dioxo-3,4-dihydropyrido[3',2': 4,5]thieno[3,2-d]pyrimidin-8-carboxylate; 33

2-Aminocarboxamide derivative **11** (0.56 g, 2 mmol) was fused in a water bath for 12 h with chloroacetyl chloride (0.22 g, 0.15 mL, 2 mmol). The reaction mixture was allowed to cool and the obtained product was filtered then washed with different solvents to yield compound **33**.

Dark brown crystals, 38 % yield, m.p.; > 360 °C; **IR** (KBr, cm^{-1}): 3327, 3292, 3170 (NH); 3051 (C-H aromatic); 2924 (C–H aliphatic); 1720, 1690, 1670 (C=O); 1604 (C=N); 1571 (C=C); 1280, 1060 (C–S–C), 1261, 1060 (C–O–C); 800 (C–Cl). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.20-1.23 (m, 3H, –OCH$_2$CH$_3$); 2.35 (s, 2H, CH$_2$Cl); 3.20-3.81 (m, 2H, –OCH$_2$CH$_3$, under DMSO); 8.29 (s, 1H, pyridine–NH, D$_2$O exchangeable); 8.42 (s, 1H, pyrimidine–NH, D$_2$O exchangeable); 8.82 (s, 1/2 H, pyridine–C$_4$–H); 11.69 (s, 1/2 H, OH tautomer, D$_2$O exchangeable). **MS** m/z (relative intensity %): 339 (M$^{+•}$, 5.96). **Anal. Form:** C$_{13}$H$_{10}$ClN$_3$O$_4$S (339). **Calcd. (%):** C, 45.96; H, 2.97; N, 12.37; S, 9.44. **Found (%):** C, 46.08, H, 2.99; N, 12.50; S, 9.59.

Ethyl 4,7-dioxo-2-thioxo-6H, 9H, 1,2,3,4-tetrahydropyrido[3',2': 4,5] thieno[3,2-d]pyrimidin-8-carboxylate; 34

A mixture of the o-aminocarboxamide derivative **11** (0.56 g, 2 mmol), carbon disulfide (0.15 g, 0.2 mL, 2 mmol) and potassium carbonate (0.28 g, 2 mmol) in dimethylformamide (5 ml) was refluxed for 20 h. The reaction mixture was then cooled, diluted with cold water and acidified with dilute acetic acid. The obtained precipitate was collected by filtration and washed with several solvent to yield compound **34**.

Brown powder, 42 % yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3379, 3336, 3186 (NH); 3055 (C-H aromatic); 2920 (C–H aliphatic); 1700, 1680 (C=O); 1597 (C=N); 1558 (C=C); 1541, 1301, 1139, 1060 (I, II, III,IV bands N–C=S); 1269, 1060 (C-S-C); 1261, 1060 (C–O–C). **^{1}H-NMR** (DMSO-d$_6$, δ ppm): 1.20-1.30 (m, 3H, –OCH$_2$CH$_3$); 3.40-3.61 (m, 2H, –OCH$_2$CH$_3$); 7.95 (s, 1H, pyridine–C$_4$–H); 8.15 (s, pyridine–NH, D$_2$O exchangeable); 9.57 (s, 1H, pyrimidine–N$_1$-H, D$_2$O exchangeable); 9.69 (s, 1H, pyrimidine–N$_3$-H, D$_2$O exchangeable). **MS** m/z (relative intensity %): 323 (M$^{+•}$, 1.33); 322 (M$^{+•}$-1, 0.73); 321 (M$^{+•}$-2, 1.05). **Anal. Form:** C$_{12}$H$_9$N$_3$O$_4$S$_2$ (323). **Calcd. (%):** C, 44.57; H, 2.81; N, 13.00; S, 19.83. **Found (%):** C, 44.72; H, 2.81; N, 13.14; S, 19.91.

Ethyl 4,7-dioxo-2(4-chlorophenyl)-6H, 9H-3,4-dihydropyrido[3',2': 4,5]thieno[3,2 –d]pyrimidine-8-carboxylate; 35

An equimolar mixture of compound **11** (0.56 g, 2 mmol) and 4-chlorobenzaldehyde (0.28 g, 2 mmol) was fused for 2 h in presence of few drops of piperidine (2-3 drops). The formed solid was collected by filtration and crystallized from dimethylformamide to give compound **35.**

Dark brown powder, 53 % yield, m.p.; > 360 °C, **IR** (KBr, cm^{-1}): 3385, 3327, 3169 (NH); 3057 (C–H aromatic); 2929 (C–H aliphatic); 1710, 1680, 1660 (C=O); 1595 (C=N); 1558 (C=C); 1269, 1089 (C–S–C & C–O–C); 1089 (p–Cl-C$_6$H$_5$). **^{1}H-NMR** (DMSO-d$_6$, δ

ppm): 1.53-1.65 (m, 3H, –OCH$_2$CH$_3$); 3.90-4.00 (m, 2H, –OCH$_2$CH$_3$); 7.20-7.50 (m, 2H, 4-Cl-C$_6$H$_4$-C$_{2,6}$-H); 7.95 (s, 1H, pyridine–C$_4$–H); 8.30-8.48 (m, 2H, 4-Cl-C$_6$H$_4$-C$_{3,5}$-H); 9.32 (s, 1H, pyridine–N$_1$–H, D$_2$O exchangeable); 9.48 (s, 1H, pyrimidine–N$_3$–H, D$_2$O exchangeable). **MS** m/z (relative intensity %): 402 (M$^{+•}$+1, 0.76); 399 (M$^{+•}$-2, 0.60); 398 (M$^{+•}$-3, 0.79). **Anal. Form:** C$_{18}$H$_{12}$ClN$_3$O$_4$S (401). **Calcd. (%):** C, 53.80; H, 3.01; N, 10.46; S, 7.98. **Found (%):** C, 54.01; H, 3.07; N, 10.57; S, 8.05.

Anticancer screening

Mammalian cell lines: MCF-7 cells (human breast cancer cell line) were obtained from VACSERA Tissue culture unit HepG-2 cells (human cell line of a well differentiated hepatocellular carcinoma isolated from a liver biopsy of a male Caucasian aged 15 years) were obtained from the American type culture collection (ATCC).

Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from sigma (st. louis, mo., USA) DMEM, RPM1-1640, FBS, HEPES buffer solution, L–glutamine, gentamycin and 0.25% trypsin-EDTA which were purchased from (Bio whittaker® Lonza, Belgium). Furthermore, crystal violet stains (1%): are prepared from 0.5% (w/v) crystal violet and 50% methanol then made up to volume with water and filtered through a Whatmann No. 1. Filter paper.

Cell line Propagation

The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/mL gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO$_2$ and were sub-cultured two times a week. Cell toxicity was monitored by determining the effect of the test samples on cell morphology and cell viability.

Cytotoxicity evaluation using viability assay

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1x10^4 cells per well in 100µL of growth medium. Fresh medium containing different concentrations of the test sample was added after 24 h. of seeding. Serial two-fold dilutions of the tested chemical compounds were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO$_2$ for a period of 48 h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the

experiment. After incubation of the cells for 24 h at 37°C, various concentrations of sample (500, 250, 125, 62.5, 31.25 & 15.6 µg) were added, and the incubation was continued for 48 h and viable cells yield was determined by a colorimetric method.

In brief, after the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 min. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 490 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated.

RESULTS AND DISCUSSION

Chemistry

Literature survey revealed that, thienopyridine derivatives were reported as potent anticancer agents [29-32]. Therefore, 5-cyano-6-mercaptopyridine **2** was fused with chloroacetone to yield the cyclic thieno[2,3-b]pyridine derivative **3** as revealed in **scheme 1.** The **IR** spectrum of compound **3** lacked the absorption band due to CN group and showed absorption bands due to NH_2 function at 3387 and 3292 cm^{-1}. The ^{1}**H-NMR** spectrum of compound **3** showed a singlet signal at δ 2.55 ppm corresponding to acetyl CH_3 protons, in addition to; a deuterium oxide exchangeable singlet signal at δ 6.38 ppm due to NH_2 protons. However, compound **2** was refluxed with an equimolar amount of chloroacetic acid in a mixing solvent of acetic anhydride and acetic acid (1:2) in presence of anhydrous sodium acetate to yield thieno[2,3-b]pyridine derivative **4.** The **IR** spectrum of compound **4** lacked the absorption band due to CN function and showed broad absorption bands due to carboxylic OH group at 3446 and 3421 cm^{-1}, in addition to; absorption bands at 3275, 3228 cm^{-1} corresponding to NH_2 and NH functions. The ^{1}**H-NMR** spectrum of compound **4** revealed two deuterium oxide exchangeable singlet signals at δ 6.13 and δ 8.15 ppm corresponding to NH_2 and pyridine-N_1–H protons; respectively. In addition to; a deuterium oxide exchangeable singlet signal at δ 11.60 ppm due to carboxylic OH proton. While**,** the reaction of compound **2** with ethyl chloroacetate in ethanolic solution of sodium ethoxide yielded both the open chain S-alkylated product **5** and the fused theino[2,3-b]pyridine derivative **6. IR** spectrum of compound **6** lacked the absorption band due to CN group and showed absorption bands at 3446, 3311 and 3201

cm^{-1} due to NH_2 and NH functions. ^{1}**H-NMR** spectrum of compound **5** revealed two singlet signals integrated for one proton and half proton at δ 4.70 and 8.32 ppm; respectively corresponding to SCH_2 and S<u>CH</u>=C–OH tautomer; respectively. In addition to, a deuterium oxide exchangeable singlet signal at δ 7.51 ppm due to SCH=C–<u>OH</u> tautomer integrated for half proton, while the ^{1}**H-NMR** spectrum of compound **6** showed an additional deuterium oxide exchangeable singlet signal at δ 7.63 ppm due to NH_2 protons. Furthermore, 5-cyano-6-mercaptopyridine **2** was refluxed with 4-methoxyphenacyl bromide in dimethylformamide in presence of potassium carbonate as a base to yield both the open chain S-alkylated derivative **7** and the fused thienopyridine derivative **8**. **IR** spectrum of compound **8** lacked the absorption band due to CN function and revealed absorption bands at 3388, 3269 and 3188 cm^{-1} due to NH_2 and NH groups. ^{1}**H–NMR** spectra of compound **7** and **8** showed singlet signals at δ 3.71 and 3.51 ppm corresponding to OCH_3 protons; respectively. The ^{1}**H-NMR** spectrum of compound **7** revealed a singlet signal at δ 4.20 ppm corresponding to S-<u>CH$_2$</u>–C=O protons. While, the ^{1}**H–NMR** spectrum of compound **8** showed a deuterium oxide exchangeable singlet signal at δ 5.21 ppm due to NH_2 protons**.**

In addition, the reaction of 5-cyano-6-mercaptopyridine **2** with chloroacetonitrile by stirring at room temperature in dimethylformamide containing potassium carbonate yielded both the S-alkylated derivative **10** and the o-aminonitrile thienopyridine derivative **9** as showed in **Scheme 2**.

However, refluxing of compound **2** with cloroacetonitrile in dimethylformamide/potassium carbonate mixture afforded only the cyclization product **9.** Compound **9** prepared by the aforementioned two conditions were found to be identical as revealed by TLC, m.p. mixed m.p and **IR** spectrum. ^{1}**H-NMR** spectrum of compound **9** revealed a deuterium oxide exchangeable singlet signal at δ 6.89 ppm due to NH_2 protons, while the ^{1}**H-NMR** spectrum of compound **10** showed a singlet signal at δ 3.47 ppm integrated for one proton due to S<u>CH$_2$</u>CN. In addition to, a deuterium oxide exchangeable singlet signal at δ 6.34 ppm integrated for half proton due to the tautomeric S-CH=C=<u>NH</u> proton and a singlet signal at δ 8.32 ppm integrated for half proton corresponding to S-<u>CH</u>=C=NH tautomer. However, upon refluxing of compound **2** with

chloroacetamide in dimethylformamide in presence of anhydrous potassium carbonate yielded the cyclic 3-amino-2-carbamoylthieno[2,3-b]pyridine derivative **11** through the formation of a non-isolated S-alkylated derivative which in turn underwent Thorpe-Zeiglar cyclization [33,34] to afford the target compound**.** ^{1}**H-NMR** spectrum of compound **11** revealed two deuterium oxide exchangeable singlet signals at δ 6.75
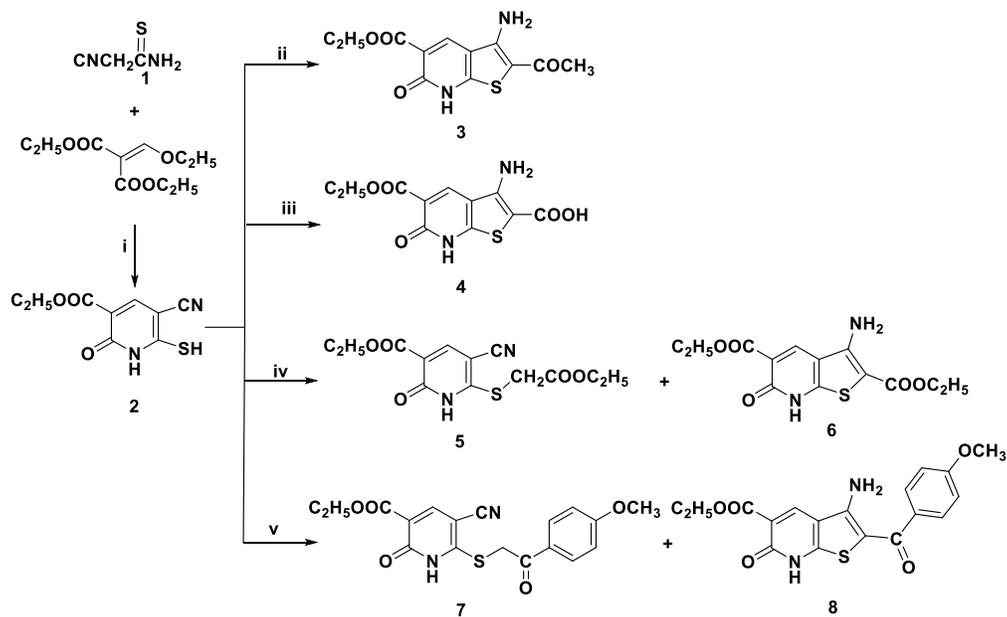
Reagents & conditions: (i) NaOC$_2$H$_5$/EtOH/R.T.; **(ii)** ClCH$_2$COCH$_3$/fusion; **(iii)** ClCH$_2$COOH/ AC$_2$O/ACOH/NaOAC/reflux; **(iv)** ClCH$_2$COOC$_2$H$_5$/NaOC$_2$H$_5$/reflux; **(v)** 4-CH$_3$O-C$_6$H$_4$-COCH$_2$Br/anh. K$_2$CO$_3$/DMF/reflux.

Scheme 1
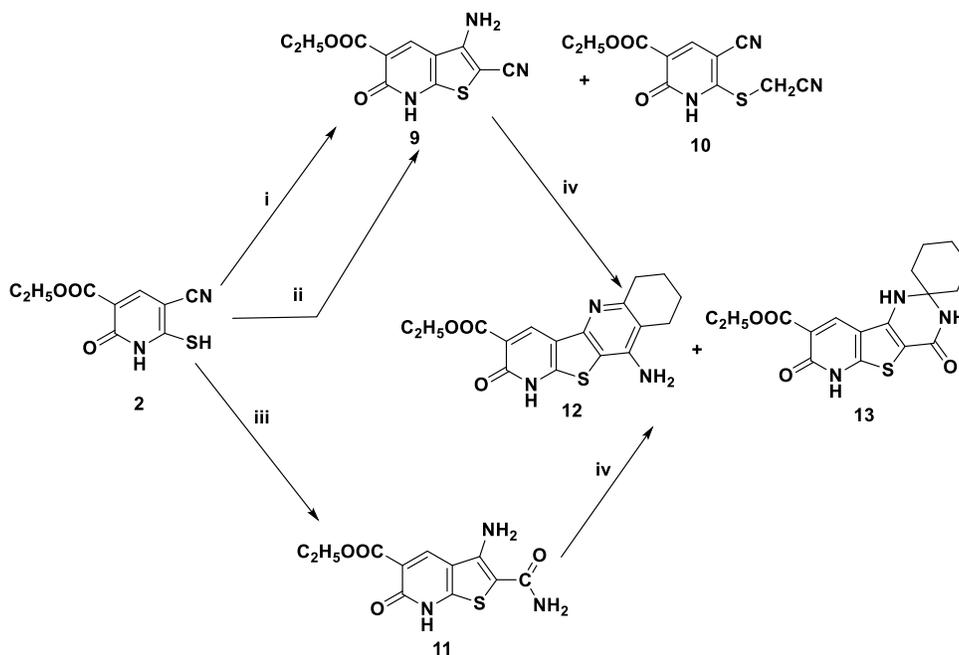
Reagents & conditions: **(i)** ClCH$_2$CN/DMF/ anh. K$_2$CO$_3$/R.T.; **(ii)** ClCH$_2$CN/DMF/ anh. K$_2$CO$_3$/reflux; **(iii)** ClCH$_2$CONH$_2$/DMF/ anhy. K$_2$CO$_3$/reflux; **(iv)** cyclohexanone/ZnCl$_2$/DMF/reflux.

Scheme 2

and 7.44 ppm corresponding to thienopyridine–C_2–NH_2 and thienopyridine–C_3–NH_2; respectively. Moreover, the target aminoquinolino derivative **12** and the spiropyrimidine analogue **13** were obtained by two methods either by refluxing of the o-aminonitrile derivative **9** or the o-aminocarboxamide compound **11** with cyclohexanone in dimethylformamide containing zinc chloride as a catalyst. The reaction mechanism for the synthesis of compounds **12** and **13** from o-aminonitrile derivative **9** was reported [35] to be achieved as follows (**Figure 2**).

The postulated reaction mechanism for the synthesis of compounds **12** and **13** from o-aminocarboxamide analogue **11** is illustrated as follows in **Figure 3**:

IR spectra of compounds **12** and **13** lacked the absorption bands due to CN group present in their precursor**.** However, the **1H-NMR** spectrum of compound **12** revealed multiplet signals at δ 1.80-1.85, 3.11-3.19 and 3.40-3.50 ppm corresponding to tetrahydroquinoline–$C_{7,8}$–CH_2, tetrahydroquinoline–C_9–CH_2 and tetrahydroquinoline–C_6–CH_2; respectively. While the **1H-NMR** spectrum of compound **13** exhibited multiplet signals due to cyclohexyl protons at their expected chemical shifts .

Furthermore, the reaction of 5-cyano-6-mercaptopyridine derivative **2** with several aliphatic methyl ketones; namely ethyl methyl ketone, cyclopentanone and acetylacetone in glacial acetic acid and acetic anhydride containing few drops of concentrated sulphuric acid yielded their corresponding thienopyridine derivatives **14, 15** and **16**; respectively as shown in **scheme 3**. The reaction was suggested to proceed through the formation of a non-isolated S-alkylated intermediate which underwent intramolecular cyclo addition of active methine proton on the cyano function to form their corresponding 3-iminothienopyridine derivatives. **IR** spectra of compounds **14, 15** and **16** lacked the absorption bands due to cyano group of their precursor. **1H-NMR** spectra of compounds **14, 15** and **16** revealed deuterium oxide exchangeable singlet signals at δ 6.89-6.90 ppm attributed to imino proton. However, stirring of compound **2** with ethyl α-chloroacetoacetate in a mixture of ethanol and dimethylformamide (4 : 1) containing aqueous potassium hydroxide yielded the open chain S-alkylated derivative **17** and the cyclic thienopyridine derivative **18**. **1H-NMR** spectra of compounds **17** and **18** revealed two singlet signals at δ 2.71 ppm attributed to the acetyl CH_3 protons. However, **1H-NMR** spectrum of compound **17** showed a singlet signal at δ 5.79 ppm due to 3-oxobutanoate-C_2-H, while, **1H-NMR** spectrum of compound **18** showed a deuterium oxide exchangeable singlet signal at δ 5.80 ppm corresponding to imino proton.

In attempts to study the effect of fusion of different heterocyclic systems on the anticancer activity of the pyridine back bone, 5-cyano-6-mercaptopyridine derivative **2** was fused with oxalyl chloride to yield both the S-acylated derivative **19** and the cyclic dioxo-thiazolopyridine derivative **20** as shown in **Scheme 4.**

IR spectra of compounds **19** and **20** showed absorption bands corresponding to carbonyl functions at 1737, 1697 and 1701, 1680, 1670 cm^{-1}; respectively. Besides, the **IR** spectrum of compound **20** lacked the absorption bands due to NH function. However, compound **2** was refluxed with ethyl chloroformate in dimethylformamide in presence of potassium carbonate as a base to yield the open chain S-alkylated derivative **21**. IR spectrum of compound **21** showed absorption bands at 1720, 1701 and 1670 cm^{-1} corresponding to three carbonyl functions. The fusion of compound **2** with dimethyl acetylene dicarboxylate yielded the thiazolopyridine derivative **22.** The reaction mechanism is suggested to proceed through the initial nucleophilic addition of the thiol function on the acetylinic carbons followed by nucleophilic attack of pyridine–N_1–NH function on carbonyl ester group with subsequent elimination of a methanol molecule to yield the thiazole derivative **22**. The postulated reaction mechanism is illustrated as follows in **Figure 4**.

IR spectrum of compound **22** lacked the absorption bands due to NH function. The target triazolopyridine derivative **24** was prepared by the reaction of compound **2** with N'-(4-chlorophenyl)-2-oxopropanehydrazonoyl chloride **23** in dimethylformamide in presence of potassium carbonate as a base. The reaction mechanism is reported [36,37] to proceed as follows in **Figure 5**.

1H-NMR spectrum of compound **24** showed a singlet signal at δ 2.89 ppm corresponding to –$COCH_3$ protons and lacked any deuterium oxide exchangeable signal due to NH proton. Furthermore, the target S-methyl analogue **25** was prepared by stirring a mixture of the thiol derivative **2** and methyl iodide in dimethyl formamide in presence of potassium hydroxide as a base. The reaction of the methylthio derivative **25** with two equivalents of hydrazine hydrate in absolute ethanol yielded the dipyrazolo[3,4-b:4',3'-e]pyridine-3(2H)-one derivative **26**. IR spectrum of compound **26** lacked the absorption bands due to cyano function and ester carbonyl group. **1H-NMR** spectrum of compound **26** revealed two deuterium oxide exchangeable singlet signals at δ 5.23 and δ 6.21 ppm due to NH_2 and two pyrazole NH protons; respectively**.**

Furthermore, the reaction of 5-cyano-6-mercaptopyridine **2** with cinnamoyl chloride in a mixture of pyridine/benzene (1:1) yielded both the open chain α,β-unsaturated carbonyl derivative **28** and the cyclic pyrido[2,1-b][1,3]thiazine derivative **29** as revealed in **Scheme 5.**
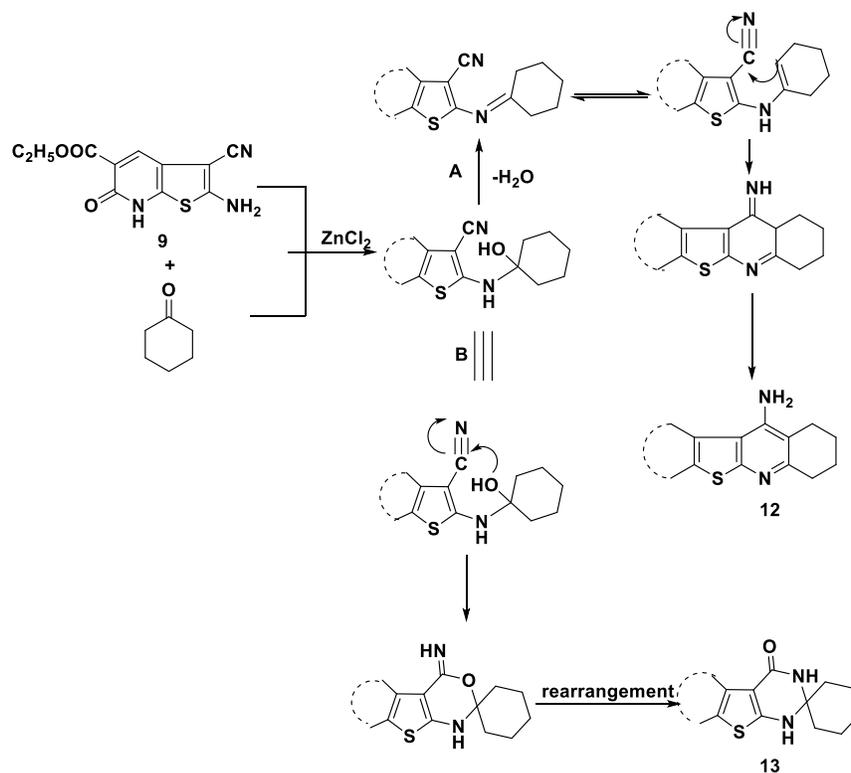
Figure 2. Mechanistic pathway for the preparation of compounds 12 and 13 from o-aminonitrile derivative 9.
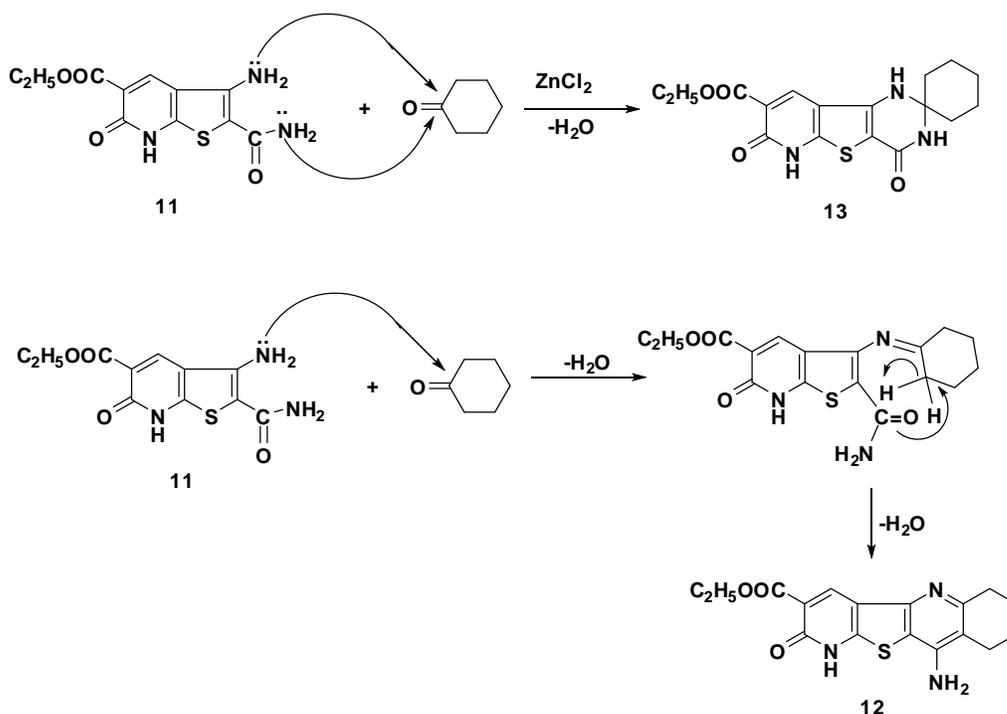
Figure 3. Mechanistic pathway for the preparation of compounds 12 and 13 from o-aminocarboxamide analogue 11.
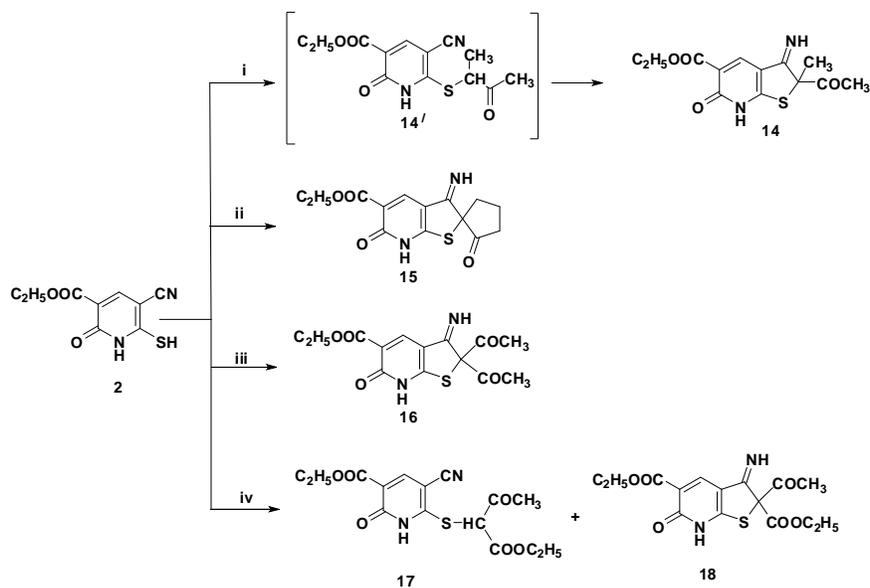
Reagents & conditions: (i) $CH_3COC_2H_5/ACOH/H_2SO_4/AC_2O/reflux$; **(ii)** cyclopentanone/ $ACOH/H_2SO_4/AC_2O/reflux$; **(iii)** $CH_3COCH_2COCH_3/ACOH/H_2SO_4/AC_2O/reflux$; **(iv)** $CH_3COCH(Cl)COOC_2H_5/EtOH/KOH/DMF/R.T.$

Scheme 3
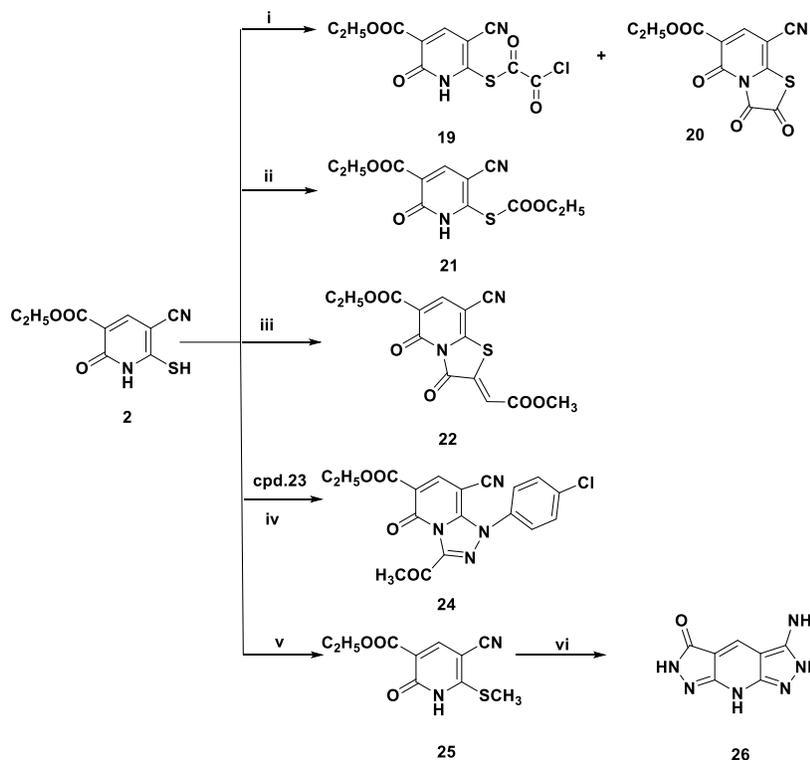
Reagents & conditions: (i) $(COCl)_2$/fusion; **(ii)** $ClCOOC_2H_5/DMF$/ anh. K_2CO_3/reflux; **(iii)** DMAD/fusion; **(iv)** $4\text{-}ClC_6H_4\text{-}NH\text{-}N=CH(Cl)COCH_3/DMF/$ anh. K_2CO_3/reflux; **(v)** $CH_3I/DMF/KOH/R.T.$; **(vi)** $NH_2NH_2/EtOH/reflux$.

Scheme 4

¹H-NMR spectrum of compound **28** showed two doublet signals at δ 6.52 and 7.59 ppm corresponding to olefinic =CH–C=O and =CH–C₆H₅ protons; respectively. While the **¹H-NMR** spectrum of compound **29** showed a multiplet signal at δ 7.36-7.40 ppm and doublet signal at δ 7.77 ppm due to thiazine-C₄-H and thiazine-C₅-H; respectively. Moreover, compound **2** was refluxed with 2,4-dimethoxybenzylidenemalononitrile **30** in dimethylformamide in presence of a catalytic amount of potassium carbonate to give the thiopyranopyridine derivative **31** and the pyridothiazine analogue **32**. The reaction mechanism is postulated to proceed through the reaction of thiol function with benzylidenemalononitrile to yield the Michael adduct which reacted either through the nucleophilic addition of the active methylene function on the 5-cyano group of pyridine with subsequent elimination of a hydrogen cyanide molecule to yield compound **31** or via the nucleophilic addition of pyridine NH function on one of the cyano groups of the benzylidenemalononitrile with the elimination of a hydrogen cyanide molecule to give compound **32**. The postulated reaction mechanism for the synthesis of compound **31** is illustrated as follows **Figure 7**:

The postulated reaction mechanism for the synthesis of compound **32** is illustrated as follows **Figure 8**:

Figure 8: Mechanistic pathway for the preparation of compound **32**.

IR spectra of compounds **31** and **32** showed absorption bands at 3211, 3190 & 3363, 3188 cm⁻¹ due to NH functions; respectively**.**

Our aim was also directed to study the biological activity of pyrimidine nucleus fused to the thienopyridine moiety in the field of anticancer agents. Therefore, the target pyridothienopyrimidinone derivative **33** was synthesized via fusion of the o-aminocarboxamide derivative **11** with chloroacetyl chloride as shown in **scheme 6**. **¹H-NMR** spectrum of compound **33** revealed a deuterium oxide exchangeable singlet signal at δ 8.42 ppm due to pyrimidine-NH proton and lacked any deuterium oxide exchangeable singlet due to NH₂ protons of its precursor. Also, the o-aminocaboxamide derivative **11** was refluxed with carbon disulfide in dimethylformamide containing potassium carbonate to give the corresponding 2-thioxothienopyrimidinone derivative **34**. **¹H-NMR** spectrum of compound **34** revealed three deuterium oxide exchangeable singlet signals at δ 8.15, 9.57 and 9.69 ppm corresponding to pyridine–NH, pyrimidine–N₁–H and pyrimidine–N₃–H protons; respectively. Finally, the fusion of 2-aminocarboxamide derivative **11** with 4-chlorobenzaldehyde in presence of piperidine yielded the corresponding dihydropyridothienopyrimidinone analogue **35**. **¹H-**NMR spectrum of compound **35** revealed two deuterium oxide exchangeable singlet signals at δ 9.32 and 9.48 ppm due to pyridine-N₁-H and pyrimidine-N₃-H protons; respectively.

Anticancer screening

Thirty three synthesized compounds (**3-35**) were screened for their *in vitro* cytotoxic activity against human hepatocellular liver carcinoma (HepG2) and human breast cancer (MCF-7) cell lines in the regional center for mycology and biotechnology, at Al-Azhar University using 5-fluorouracil (5-FU) as the reference drug.

IC₅₀ values and the six dose growth inhibition percent of the tested compounds (**3-35**) against liver HepG2 and breast MCF-7 cell lines are represented in tables (**1,2**); respectively.

Table (1): IC₅₀ values and six dose growth inhibition percent of the tested compounds against liver HepG2 cell line.

As revealed from the results presented in tables (**1,2**) and in a trial to shed more light on the SAR of compounds bearing ethyl 6-oxothienopyridine-5-carboxylate backbone possessing different substituents in the 2-position, it is evident that the presence of a ketone function at 2-position as an acetyl group in compound **3** or 4-methoxybenzoyl as in compound **8** exhibited moderate to weak anticancer activities against both HepG2 and MCF-7 cell lines. However, replacement of the ketonic function by a carboxylic group as in compound **4** resulted in marked increase in its anticancer activities against both HepG2 and MCF-7 cell lines which is more potent than the reference drug 5-fluorouracil. Furthermore, the esterification of the carboxylic group as in compound **6** resulted in nearly equipotent activity to 5-fluorouracil against HepG2 cell line while it diminished the activity against MCF-7 cell line. However, amidation of the carboxylic group as in compound **11** or replacing the carboxylic function by cyano group as in compound **9** abolished the anticancer activities against both cell lines. Moreover, concerning the activities of different S-substituted ethyl 5-cyano-2-oxo-6-thioxopyridine-3-carboxylate backbone as in compounds **5, 10** and **21** bearing; 2-ethoxy-2-oxoethylthio, cyanomethylthio and ethoxycarbonylthio side chains; respectively exihibited nearly equipotent anticancer activities against HepG2 cell line compared to the reference drug with IC₅₀ values. However, compound **21** exerted two folds more potent activity against MCF-7 cell line, while compounds **5** and **10** showed moderate activities against MCF-7 cell line. Furthermore, compound **7** possessing 4-methoxybenzoylmethylthio side chain exerted moderate activities against both HepG2 and MCF-7 cell lines. It is worth mentioning that, S-oxalylchloride side chain as in compound **19** and S-cinnamoyl side chain as in compound **28** resulted in
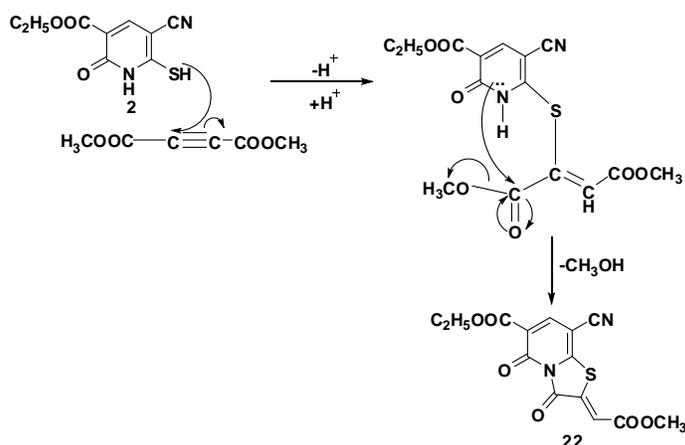
Figure 4. Mechanistic pathway for the preparation of compound 22.
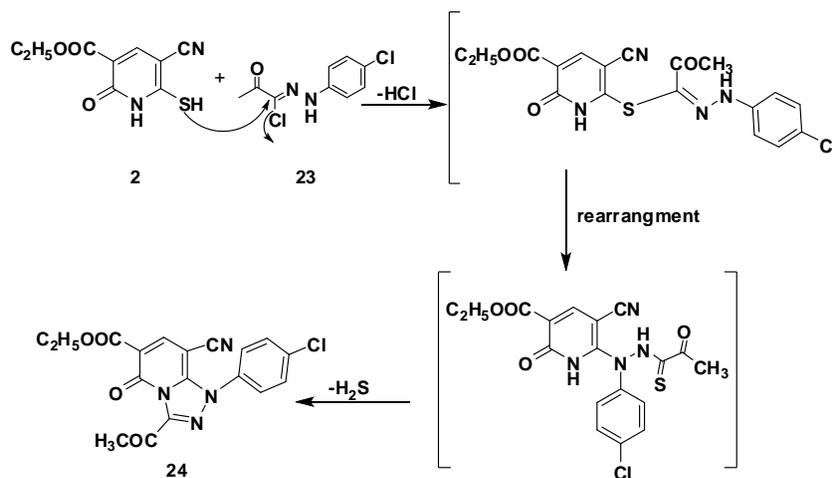
Figure 5. Mechanistic pathway for the preparation of compound 24.
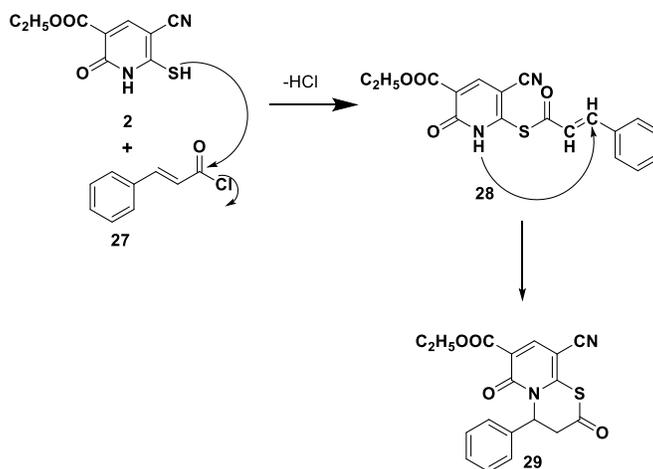
Figure 6. The postulated mechanism for synthesis of compound 28 and 29.
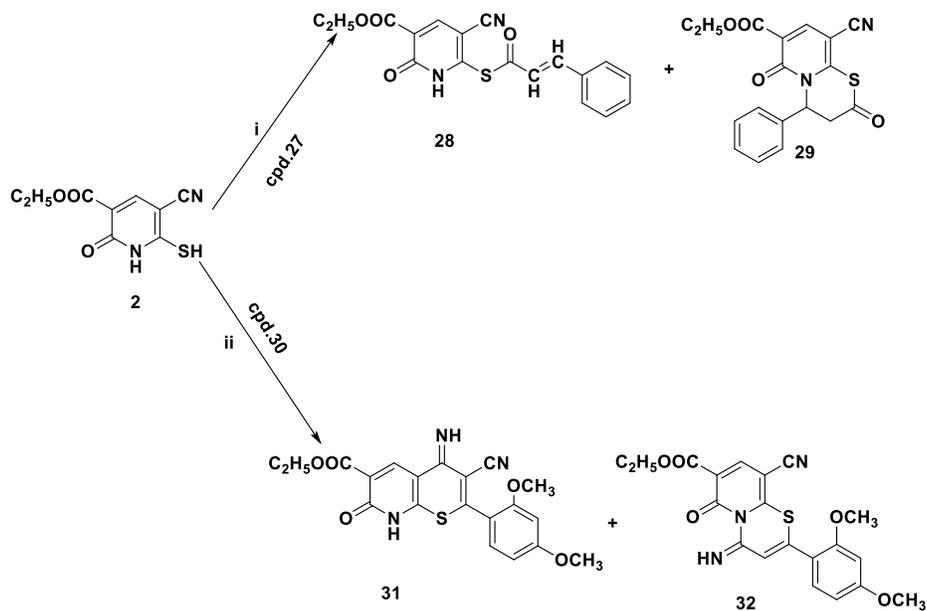
Reagents & conditions: (i) C_6H_5-CH=CHCOCl/pyridine/benzene/reflux;
(ii) 2-(2,4-dimethoxybenzylidene)malononitrile/ DMF/ anh. K_2CO_3/reflux.

Scheme 5
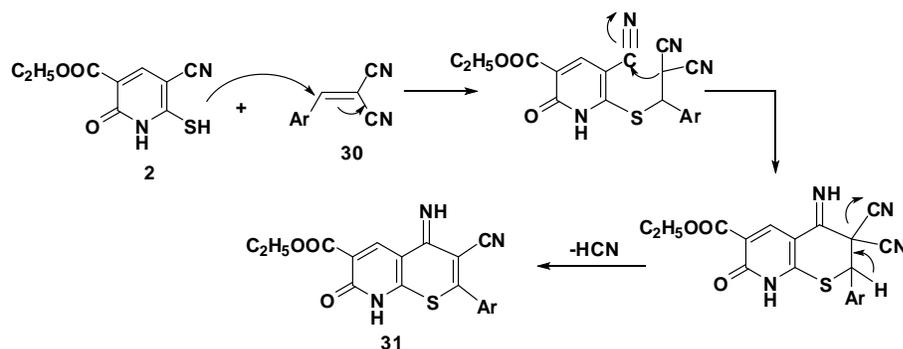
Figure 7. Mechanistic pathway for the preparation of compound 31.
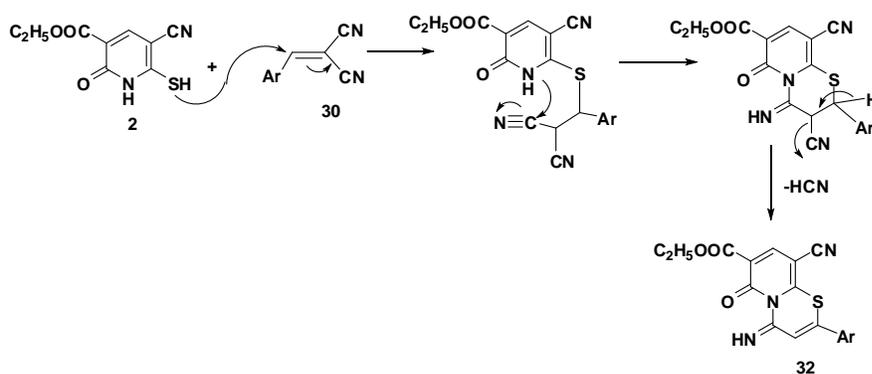
Figure 8. Mechanistic pathway for the preparation of compound 32.
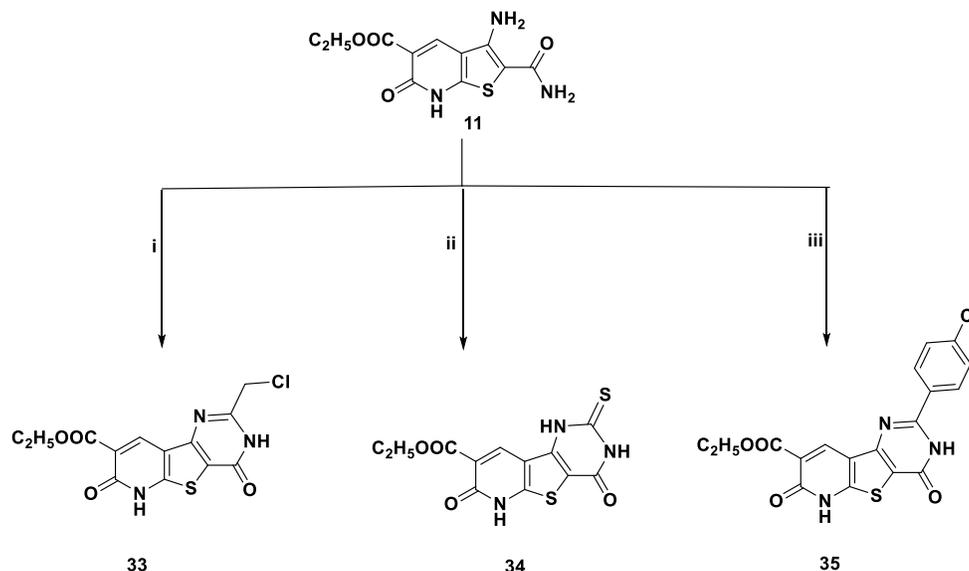
Reagents & conditions: (i) ClCH$_2$COCl/fusion; **(ii)** CS$_2$/ anh. K$_2$CO$_3$/DMF/reflux;
(iii) 4-Cl-C$_6$H$_4$-CHO/pipridine/fusion.

Scheme 6

marked improvement in activity against both cell lines with two folds more potent activities than the reference drug. Moreover, the presence of ethyl 3-oxobutanoate side chain as in compound **17** diminished the anticancer activities against both HepG2 and MCF-7 cell lines, while the S-methyl side chain in compound **25** abolished the activity against HepG2 cell line while it exerted moderate activity against MCF-7 cell line. In a trial to investigate the effect of fusion of different rings to ethyl 6-oxothienopyridine-5-carboxylate backbone, it was found that, fusion of 4-aminotetrahydroquinoline to the ethyl 6-oxothienopyridine-5-carboxylate backbone as in compound **12** resulted in highly potent anticancer agent against both HepG2 and MCF-7 cell lines this might be an indication of the better binding to the protein . While fusion of 2-substituted-4-oxopyrimidine moieties to the ethyl 6-oxothienopyridine-5-carboxylate backbone as in compounds **13, 33, 34** and **35** showed variable activities. However, the replacement of spirocyclohexyl ring with chloromethyl group, thione function or 4-chlorophenyl moiety as in compounds **33, 34** and **35**; respectively, abolished the anticancer activities. Moreover, modification of the 5-amino function of ethyl 6-oxothienopyridine-5-carboxylate backbone into imino group as in compounds **14, 15, 16** and **18** bearing different substituents in 6-position resulted in inactive compounds except for compound **18** bearing acetyl and ethyl carboxylate groups in 6-position exhibited good anticancer activity against MCF-7 cell line. Furthermore, concerning the various activities of different rings fused

to the ethyl 5-cyano-2-oxopyridine-3-carboxylate backbone, it was found that, the fusion of substituted thiazole ring to the ethyl 5-cyano-2-oxopyridine-3-carboxylate backbone as in compounds **20** and **22** diminished the anticancer activities against both HepG2 and MCF-7 cell lines, while fusion of thiazine ring to the ethyl 5-cyano-2-oxopyridine-3-carboxylate backbone as in compounds **29** and **32** resulted in marked increase in the anticancer activities against both cell lines. However, fusion of [1,2,4]triazole ring to the ethyl 5-cyano-2-oxopyridine-3-carboxylate backbone as in compound **24** resulted in marked elevation in the anticancer activity against MCF-7 cell line but showed weak anticancer activity against HepG2 cell line. Whereas, the fusion of thiopyran ring to the ethyl 2-oxopyridine-3-carboxylate nucleus as in compound **31** led to nearly equipotent anticancer activity against HepG2 and half potent activity against MCF-7 cell lines compared to the reference drug 5-fluorouracil. On the other hand, fusion of two pyrazole rings to the pyridine nucleus as in compound **26** resulted in poor anticancer activities against both HepG2 and MCF-7 cell lines.

CONCLUSION

It can be concluded that most of the synthesized compounds showed strong anticancer activities against both liver cancer HepG2 and breast cancer MCF-7 cell lines. However, compounds **4, 5, 6, 10, 12, 13, 19, 21, 28, 29** and **31** exhibited more potent to equipotent

Table 1. IC$_{50}$ values and six dose growth inhibition percent of the tested compounds against liver HepG2 cell line

Compound No.	Sample concentration (µg/mL)						IC$_{50}$ (µg/mL)
	50	25	12.5	6.25	3.13	1.6	
	Growth inhibition %						
3	65.25	51.93	24.41	10.82	3.36	1.59	24.10
4	91.22	86.53	76.11	60.55	41.36	16.43	4.53
5	89.13	80.82	61.40	18.77	7.66	1.79	10.80
6	74.14	61.28	53.63	45.28	35.09	23.32	9.78
7	78.13	60.92	40.29	15.08	3.68	0.00	18.40
8	51.07	19.66	7.33	3.85	0.00	0.00	49.10
9	31.28	13.44	4.13	0.92	0.00	0.00	>50
10	86.33	70.54	56.09	21.66	7.44	1.83	11.40
11	52.11	15.54	6.19	1.28	0.00	0.00	48.60
12	85.83	76.36	68.95	43.18	19.26	8.29	7.90
13	91.59	86.26	81.04	65.52	43.04	21.51	4.09
14	69.41	58.33	42.86	26.11	9.29	4.16	18.30
15	53.19	17.57	6.11	2.02	0.00	0.00	47.80
16	18.42	5.84	1.26	0.00	0.00	0.00	>50
17	65.47	47.02	16.44	8.28	2.16	0.00	29
18	61.48	48.33	16.96	5.84	1.63	0.00	28.2
19	91.51	85.07	78.18	71.41	50.64	21.28	3.09
20	52.14	20.38	8.42	2.66	0.00	0.00	48.30
21	88.46	80.38	64.57	23.98	10.79	3.54	10.30
22	19.37	5.26	0.85	0.00	0.00	0.00	>50
24	68.16	42.85	21.60	10.71	4.22	1.54	32.10
25	70.88	32.62	10.59	3.61	0.98	0.00	36.40
26	60.47	37.26	20.14	8.53	1.87	0.00	38.70
28	91.26	84.52	73.27	69.49	51.38	20.87	3.05
29	80.87	68.26	56.05	25.42	8.58	2.84	11.30
31	85.27	68.92	57.41	43.32	18.51	4.77	9.21
32	83.43	67.06	51.25	18.33	5.61	0.43	12.30
33	59.13	20.48	8.32	3.06	0.92	0.00	44.10
34	63.07	17.31	8.57	1.55	0.00	0.00	42.90
35	73.61	54.52	20.48	8.36	1.59	0.00	23.30
5-FU	81.07	68.38	55.83	34.69	26.52	18.78	10.80

Table 2. IC$_{50}$ values and six dose growth inhibition percent of the tested compounds against breast MCF-7 cell line

Compound No.	Sample concentration (µg/mL)						IC$_{50}$ (µg/mL)
	50	25	12.5	6.25	3.13	1.6	
	Growth inhibition %						
3	74.62	62.16	34.68	15.68	1.83	0.00	19.50
4	88.51	74.09	63.18	51.23	29.72	12.31	6.07
5	71.79	58.08	43.82	28.38	12.66	7.35	17.90
6	68.53	54.74	31.93	9.87	3.71	1.24	22.40
7	75.49	61.27	35.04	10.52	3.76	1.22	19.60
8	63.46	40.54	19.05	8.31	1.99	0.00	35.30
9	40.43	21.88	8.56	2.15	0.00	0.00	>50
10	81.58	68.41	52.96	30.77	13.83	9.16	11.70
11	58.35	37.13	23.08	10.85	3.68	1.46	40.20
12	89.53	81.48	70.27	65.72	56.11	18.41	2.87
13	90.22	82.75	68.54	57.21	35.64	18.72	5.21
14	60.15	39.03	12.54	3.95	1.28	0.00	38.00
15	65.38	18.07	7.16	1.83	0.00	0.00	41.90
16	29.34	12.98	5.87	1.06	0.00	0.00	>50
17	59.03	30.87	12.48	5.82	1.31	0.00	42.00
18	81.06	68.92	57.53	33.06	18.28	7.02	10.60
19	86.28	78.41	59.37	57.25	42.16	19.09	4.75
20	54.28	35.82	19.02	10.24	4.86	0.77	44.20
21	93.11	88.58	79.33	68.72	51.04	28.16	3.05
22	28.18	11.21	5.87	0.92	0.00	0.00	>50
24	83.74	74.26	61.04	45.61	19.09	10.28	8.03
25	76.83	67.95	52.69	18.04	9.66	4.72	12.00
26	51.02	41.26	23.92	14.07	6.28	0.85	38.90
28	90.38	84.32	71.91	50.17	15.02	8.58	6.23
29	90.35	84.17	75.68	67.61	25.74	10.69	4.94
31	80.47	63.92	54.84	21.55	9.74	2.69	11.60
32	79.26	68.17	56.52	17.06	9.69	2.72	11.50
33	62.78	39.22	23.06	10.73	3.92	1.29	36.40
34	59.87	30.71	13.94	4.88	1.26	0.00	41.50
35	53.71	26.38	12.66	4.75	1.29	0.00	46.60
5-FU	92.13	83.02	65.35	47.17	31.83	19.58	7.22

activities against HepG2 cell line with IC_{50} values ranging from 3.05-11.40 µg/mL compared to the reference drug 5-fluorouracil (IC_{50} value 10.80 µg/mL). While compounds **4, 12, 13, 19, 21, 24, 28** and **29** were the more potent to equipotent against MCF-7 cell line exerting IC_{50} values ranging from 2.87-6.23 µg/mL compared to the reference drug 5-fluorouracil (IC_{50} value 7.22 µg/mL). It is worth mentioning that, compounds **4, 12, 13, 19, 21, 28** and **29** exerted highly potent anticancer activities against both HepG2 and MCF-7 cell lines.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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