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Research Article

Synthesis of Some Polysubstituted Nicotinonitriles and Derived Pyrido[2,3-*d*]pyrimidines as *In Vitro* Cytotoxic and Antimicrobial Candidates

Hassan M. Faidallah,[1,2] Sherif A. F. Rostom,[3,4] and Khalid A. Khan[1]

[1]*Department of Chemistry, Faculty of Science, King Abdulaziz University, P.O. Box 80203, Jeddah 21589, Saudi Arabia*
[2]*Department of Chemistry, Faculty of Science, University of Alexandria, Alexandria, Egypt*
[3]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, King Abdulaziz University, P.O. Box 80260, Jeddah 21589, Saudi Arabia*
[4]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria 21521, Egypt*

Correspondence should be addressed to Hassan M. Faidallah; hfaidallahm@hotmail.com

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The synthesis of polysubstituted pyridines, in addition to some derived pyrido[2,3-*d*]pyrimidine ring systems supported with chemotherapeutically active functionalities, is described. They were evaluated for their *in vitro* cytotoxic effects against three different human tumor cell lines (human colon carcinoma HT29, hepatocellular carcinoma Hep-G2, and Caucasian breast adenocarcinoma MCF7). Nine compounds displayed variable cytotoxic potential, among which alkylthio analogs **33**, **34**, and **37** emerged as the most active members, being almost twice as active as doxorubicin against the colon carcinoma HT29 cell line. In addition, the same three analogs showed a clear differential cytotoxic profile as they exhibited a marginal inhibitory effect on the growth of the normal nontransformed human foreskin fibroblast Hs27 cell line. Meanwhile, nineteen compounds were able to exhibit significant antibacterial activity against both Gram-positive and Gram-negative bacteria, together with moderate antifungal activities. The pyrido[2,3-*d*]pyrimidine-2(1*H*)-thione **30** together with its alkylthio derivatives **33** and **34** stemmed as the most active antimicrobial members being equipotent to ampicillin against *S. aureus*, *E. coli,* and *P. aeruginosa,* together with a noticeable antifungal activity against *C. albicans.* Compounds **33** and **34** could be considered as a promising template for possible dual antimicrobial-anticancer candidates.

1. Introduction

Over the past two decades, several reports have discussed the growing number of cancerous diseases and the prominent role displayed by chemotherapy as the most fruitful treatment for many disseminated types of tumors. This has resulted in the activation of much research targeting the discovery of prominent lead structures that would be beneficial in styling novel antitumor chemotherapeutic agents [1–3]. Additionally, the dilemma of multi-drug resistant (MDR) bacteria arising from the misuse of traditional antimicrobial agents has been extensively studied [4]. In addition, mycotic invasions became a complicated problem especially in patients who experience immunotherapy, organ transplantation, carcinomas, or AIDS [5]. Therefore, there is a genuine demand for finding out new agents endowed with antimicrobial activity that acts with nonconventional mechanisms. Patients who undergo cancer chemotherapy are clearly liable to microbial infections owing to the subsidiary reduction of immunity. Concomitant use of several drugs for treating cancer joined with microbial invasions might cause additional complications in patients with malfunctioned kidneys. Consequently, the idea of administering one drug with dual action could be beneficial from both curative and economic viewpoints. In recent years, cyanopyridines and some of their fused-ring systems such as quinolines and pyridopyrimidines have

been under focus as potential chemotherapeutic candidates because of their reported distinctive antimicrobial [6–8], antitubercular [9], and antiviral [10] activities, in addition to the well documented antineoplastic [11], antiproliferative [12], and cytotoxic [13] potentials. Furthermore, several pyridine-containing compounds were reported to possess dual anticancer and antimicrobial activities [14–17].

During our current interest in exploring new lead structures that possess chemotherapeutic potential, much attention was focused on the antimicrobial and anticancer activities of some pyridine derivatives [18–24], particularly those possessing the 4,6-disubstituted-2-aminopyridin-3-carbonitrile (nicotinonitriles) scaffold which revealed favourable broad spectrum anticancer and/or antimicrobial efficacy. The collected results persuaded the design and synthesis of novel polysubstituted nicotinonitriles and some derived pyrido[2,3-*d*]pyrimidines incorporating different functional groups that are claimed to account for the bioactivity of related antitumor and antimicrobial candidates. Additionally, the substituents encountered in these derivatives were chosen so as to affect the electronic environment of the compounds, which would synergistically influence the targeted biological activities, in an attempt to obtain novel candidates with both anticancer and antimicrobial potentials.

2. Experimental

2.1. Chemistry. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. The infrared (IR) spectra were recorded on Shimadzu FT-IR 8400S infrared spectrophotometer using the KBr pellet technique. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-400 FT NMR spectrometer using Tetramethylsilane (TMS) as the internal standard and DMSO-d_6 as a solvent (Chemical shifts in δ, ppm). Splitting patterns were designated as follows: s: singlet; t: triplet; q: quartet; m: multiplet. The Electron Impact Ionization Mass Spectra were recorded on a Nermag R10-10C at 70 ev. 1000 Ex spectrometer. Elemental analyses were performed on a 2400 Perkin Elmer Series 2 analyzer and the values found were within ±0.4% of the theoretical values. Follow-up of the reactions and checking the homogeneity of the compounds were made by TLC on silica gel-protected aluminum sheets (Type 60 F254, Merck) and the spots were detected by exposure to UV-lamp at λ 254.

*2.1.1. 2-Amino-4,6-disubstituted Nicotinonitriles (**1**–**5**).* A one-pot mixture of the appropriate aromatic aldehyde (10 mmol), the ketone (10 mmol), malononitrile (0.66 g, 10 mmol), and ammonium acetate (6.2 g, 80 mmol) in absolute ethanol (50 mL) was refluxed for 4-5 h. The reaction mixture was allowed to attain room temperature and the resulting precipitate was filtered, washed with water, dried, and recrystallized from ethanol.

*(1) 2-Amino-4-(benzo[d][1,3]dioxol-5-yl)-6-phenylnicotinonitrile (**1**).* Yield: (1.7 g, 67%); m.p.: 162–164°C; IR (cm^{-1}, KBr): 3149, 3354 (NH_2), 2218 (CN). ^{1}H NMR (δ, ppm): 5.98 (s, 2H, CH_2), 6.17 (s, 2H, NH_2), 6.74–7.88 (m, 9H, 8 Ar-H + pyridine C_5-H). ^{13}C NMR (δ, ppm): 91.4 (CH_2), 113.4, 115.6, 117.5 (CN), 120.3, 124.8, 126.6, 127.1, 128.4, 129.5, 139.9, 142.5, 148.1, 150.6, 161.5, 165.7 (Ar-C). MS: *m/z* 315 (M^+, 100), 303 (21), 289 (82), 277 (14), 273 (76), 272 (42), 227 (55), 84 (58), 75 (28). Anal.% Calcd for $C_{19}H_{13}N_3O_2$ (315.33): C, 72.37; H, 4.16; N, 13.33. Found: C, 72.25; H, 4.20; N, 13.21.

*(2) 2-Amino-6-phenyl-4-(2-thienyl)nicotinonitrile (**2**).* Yield: (1.8 g, 54%); m.p.: 190–192°C; IR (cm^{-1}, KBr): 3167, 3387 (NH_2), 2228 (CN). ^{1}H NMR (δ, ppm): 6.10 (s, 2H, NH_2), 7.02–8.11 (m, 9H, 8 Ar-H + pyridine C_5-H). ^{13}C NMR (δ, ppm): 90.6, 110.0, 118.1 (CN), 122.3, 125.2, 126.8, 127.1, 128.3, 129.6, 139.7, 142.6, 150.7, 162.2, 165.4 (Ar-C). Anal.% Calcd for $C_{16}H_{11}N_3S$ (277.35): C, 69.29; H, 4.00; N, 15.15. Found: C, 69.21; H, 4.40; N, 15.21.

*(3) 2-Amino-4-(benzo[d][1,3]dioxol-5-yl)-6-(4-bromophenyl)nicotinonitrile (**3**).* Yield: (1.9 g, 68%); m.p.: 198–200°C; IR (cm^{-1}, KBr): 3175, 3358 (NH_2), 2223 (CN). ^{1}H NMR (δ, ppm): 6.03 (s, 2H, CH_2), 6.21 (s, 2H, NH_2), 7.68–7.87 (m, 8H, 7 Ar-H + pyridine C_5-H). ^{13}C NMR (δ, ppm): 91.2 (CH_2), 113.6, 115.4, 117.8 (CN), 121.6, 125.5, 126.3, 127.0, 128.2, 129.2, 139.4, 142.2, 148.0, 150.5, 162.5, 164.9 (Ar-C). Anal.% Calcd for $C_{19}H_{12}BrN_3O_2$ (394.23): C, 57.89; H, 3.07; N, 10.66. Found: C, 57.82; H, 3.03; N, 10.61.

*(4) 2-Amino-6-(4-bromophenyl)-4-(2-thienyl)nicotinonitrile (**4**).* Yield: (2.02 g, 73%); m.p.: 200–202°C; IR (cm^{-1}, KBr): 3416, 3198 (NH_2), 2220 (CN). ^{1}H NMR (δ, ppm): 6.18 (s, 2H, NH_2), 7.05–8.01 (m, 8H, 7 Ar-H + pyridine C_5-H). ^{13}C NMR (δ, ppm): 91.4, 110.9, 118.0 (CN), 122.2, 125.3, 126.4, 127.2, 128.4, 129.3, 139.8, 142.7, 151.2, 162.8, 165.5 (Ar-C). Anal.% Calcd for $C_{16}H_{10}BrN_3S$ (356.24): C, 53.95; H, 2.83; N, 11.80. Found: C, 53.88; H, 2.79; N, 11.85.

*(5) 2-Amino-6-(4-methoxyphenyl)-4-(2-thienyl)nicotinonitrile (**5**).* Yield: (1.63 g, 62%); m.p.: 160–162°C; IR (cm^{-1}, KBr): 3172, 3382 (NH_2), 2228 (CN). ^{1}H NMR (δ, ppm): 3.75 (s, 3H, OCH_3), 6.18 (s, 2H, NH_2), 7.10–8.12 (m, 8H, 7 Ar-H + pyridine C_5-H). ^{13}C NMR (δ, ppm): 56.1 (OCH_3), 97.9, 110.0, 117.8 (CN), 122.1, 125.4, 126.4, 127.2, 128.1, 129.2, 138.9, 142.2, 151.8, 161.2, 165.4 (Ar-C). MS: *m/z* 307 (M^+, 100), 225 (18), 281 (85), 265 (78), 182 (63), 84 (15), 83 (12), 75 (34). Anal.% Calcd for $C_{17}H_{13}N_3OS$ (307.37): C, 66.43; H, 4.26; N, 13.67. Found: C, 66.08; H, 3.90; N, 13.34.

*2.1.2. 5,7-Disubstituted Pyrido[2,3-d]pyrimidine-4(3H)-ones (**6**–**10**).* A mixture of the appropriate nicotinonitriles **1**–**5** (10 mmol) and formic acid (5 mL) was heated in a boiling water bath for 30 min. After being cooled to room temperature, the reaction mixture was poured onto ice-cold water; the precipitated solid product was filtered, washed with water, dried, and recrystallized from the appropriate solvent.

*(1) 5-(Benzo[d][1,3]dioxol-5-yl)-7-phenylpyrido[2,3-d]pyrimidine-4(3H)-one (**6**).* Yield: (1.8 g, 59%; methanol); m.p.: 168–170°C; IR (cm^{-1}, KBr): 3285 (NH), 1648 (C=O). ^{1}H NMR (δ, ppm): 6.18 (s, 2H, CH_2); 6.84–7.95 (m, 10 H, Ar-H); 8.16 (s,

1H, NH). ^{13}C NMR (δ, ppm): 91.8 (CH_2), 113.5, 116.5, 118.2, 120.1, 120.2, 122.8, 125.3, 127.5, 131.4, 142.6, 144.2, 148.1, 151.9, 157.6, 164.3, 173.7 (Ar-C), 170.5 (CO). MS: *m/z* 343 (M^+, 90), 342 (100), 331 (32), 326 (20), 314 (18), 297 (44), 284 (53), 206 (10), 83 (12), 75 (22). Anal.% Calcd for $C_{20}H_{13}N_3O_3$ (343.34): C, 69.97; H, 3.82; N, 12.24. Found: C, 70.02; H, 3.77; N, 12.31.

*(2) 7-Phenyl-5-(2-thienyl)pyrido[2,3-d]pyrimidine-4(3H)-one (**7**).* Yield: (1.9 g, 62%, ethanol); m.p.: 160–162°C; IR (cm^{-1}, KBr): 3292 (NH), 1656 (C=O). ^{1}H NMR (δ, ppm): 6.76–8.02 (m, 10H, Ar-H); 8.16 (s, 1H, NH). ^{13}C NMR (δ, ppm): 117.2, 121.6, 122.2, 122.8, 125.3, 125.4, 127.5, 138.4, 142.6, 144.3, 151.9, 157.8, 164.5, 173.4 (Ar-C), 170.2 (CO). Anal.% Calcd for $C_{17}H_{11}N_3OS$ (305.36): C, 66.87; H, 3.63; N, 13.76. Found: C, 66.80; H, 3.65; N, 13.81.

*(3) 5-(Benzo[d][1,3]dioxol-5-yl)-7-(4-bromophenyl)pyrido[2,3-d]pyrimidine-4(3H)-one (**8**).* Yield: (2.3 g, 72%, ethanol); m.p.: 110–112°C; IR (cm^{-1}, KBr): 3320 (NH), 1652 (C=O). ^{1}H NMR (δ, ppm): 6.11 (s, 2H, CH_2); 6.80–7.98 (m, 9H, Ar-H); 8.21 (s, 1H, NH). ^{13}C NMR (δ, ppm): 91.5 (CH_2), 113.2, 116.7, 119.3, 120.1, 121.7, 122.8, 125.5, 127.2, 131.6, 142.3, 144.0, 148.2, 151.7, 157.3, 164.0, 173.6 (Ar-C), 170.2 (CO). Anal.% Calcd for $C_{20}H_{12}BrN_3O_3$ (422.24): C, 56.89; H, 2.86; N, 9.95. Found: C, 56.82; H, 2.79; N, 9.91.

*(4) 7-(4-Bromophenyl)-5-(2-thienyl)pyrido[2,3-d]pyrimidine-4(3H)-one (**9**).* Yield: (2.0 g, 70%, methanol); m.p.: 128–130°C; IR (cm^{-1}, KBr): 3298 (NH), 1650 (C=O). ^{1}H NMR (δ, ppm): 6.72–7.69 (m, 9H, Ar-H); 8.22 (s, 1H, NH). ^{13}C NMR (δ, ppm): 118.5, 120.6, 121.7, 122.8, 125.3, 127.5, 129.4, 132.3, 138.4, 142.3, 147.3, 157.8, 162.5, 173.1 (Ar-C), 170.3 (CO). Anal.% Calcd for $C_{17}H_{10}BrN_3OS$ (384.25): C, 53.14; H, 2.62; N, 10.94. Found: C, 53.03; H, 2.60; N, 11.03.

*(5) 7-(4-Methoxyphenyl)-5-(2-thienyl)pyrido[2,3-d]pyrimidine-4(3H)-one (**10**).* Yield: (1.8 g, 66%, ethanol); m.p.: 166–168°C; IR (cm^{-1}, KBr): 3304 (NH), 1654 (C=O). ^{1}H NMR (δ, ppm): 3.82 (s, 3H, OCH_3); 6.92–8.01 (m, 9H, Ar-H); 8.02 (s, 1H, NH). ^{13}C NMR (δ, ppm): 56.1 (OCH_3), 114.5, 118.5, 120.7, 122.8, 125.5, 127.4, 128.1, 132.2, 142.4, 147.5, 157.7, 160.6, 163.8, 173.6 (Ar-C), 170.2 (CO). MS: *m/z* 335 (M^+, 89), 334 (100), 318 (62), 304 (48), 252 (31), 206 (14), 132 (22), 83 (16), 75 (21). Anal.% Calcd for $C_{18}H_{13}N_3O_2S$ (335.38): C, 64.46; H, 3.91; N, 12.53. Found: C, 64.61; H, 3.59; N, 12.44.

*2.1.3. 2-Methyl-5,7-disubstituted-pyrido[2,3-d]pyrimidine-4(3H)-ones (**11**–**15**).* A mixture of the starting nicotinonitriles **1**–**5** (10 mmol), acetic anhydride (5 mL), and conc. H_2SO_4 (0.5 mL) was heated in a boiling water bath for 10 min and then cooled. The reaction mixture was poured onto ice-cold water, treated with 20% NaOH solution until it acquires pH of 11-12. The crude solid product was filtered, dried, and recrystallized from the appropriate solvent.

*(1) 5-(Benzo[d][1,3]dioxol-5-yl)-2-methyl-7-phenylpyrido[2,3-d]pyrimidine-4(3H)-one (**11**).* Yield: (2.42 g, 60%, methanol); m.p.: 238–240°C; IR (cm^{-1}, KBr): 3277 (NH), 1656 (C=O). ^{1}H NMR (δ, ppm): 2.35 (s, 3H, CH_3), 6.18 (s, 2H, CH_2); 6.74–7.88 (m, 9H, Ar-H); 8.16 (s, 1H, NH). ^{13}C NMR (δ, ppm): 20.9 (CH_3), 90.9 (CH_2), 113.4, 116.2, 118.1, 120.0, 121.2, 122.7, 125.4, 127.3, 131.2, 142.5, 144.1, 148.1, 151.8, 157.3, 164.1, 173.0 (Ar-C), 170.1 (CO). Anal.% Calcd for $C_{21}H_{15}N_3O_3$ (357.37): C, 70.58; H, 4.23; N, 11.76. Found: C, 70.47; H, 4.19; N, 11.85.

*(2) 2-Methyl-7-phenyl-5-(2-thienyl)pyrido[2,3-d]pyrimidine-4(3H)-one (**12**).* Yield: (2.11 g, 62%, ethanol); m.p.: 228–230°C; IR (cm^{-1}, KBr): 3270 (NH), 1654 (C=O). ^{1}H NMR (δ; ppm): 2.28 (s, 3H, CH_3), 7.25–8.09 (m, 9H, Ar-H); 8.14 (s, 1H, NH). ^{13}C NMR (δ, ppm): 21.1 (CH_3), 117.5, 121.4, 122.2, 122.8, 125.5, 125.6, 127.1, 138.5, 142.4, 144.5, 151.8, 157.7, 164.2, 173.4 (Ar-C), 168.8 (CO). MS: *m/z* 319 (M^+, 82), 318 (100), 304 (78), 302 (35), 236 (12), 206 (14), 132 (28), 84 (43), 83 (12), 75 (21). Anal.% Calcd for $C_{18}H_{13}N_3OS$ (319.38): C, 67.69; H, 4.10; N, 13.16. Found: C, 67.54; H, 4.02; N, 13.11.

*(3) 5-(Benzo[d][1,3]dioxol-5-yl)-7-(4-bromophenyl)-2-methylpyrido[2,3-d]pyrimidine-4(3H)-one (**13**).* Yield: (2.10 g, 64%, ethanol); m.p.: 220–222°C; IR (cm^{-1}, KBr): 3265 (NH), 1651 (C=O). ^{1}H NMR (δ, ppm): 2.32 (s, 3H, CH_3), 6.16 (s, 2H, CH_2); 6.82–7.96 (m, 8H, Ar-H); 8.23 (s, 1H, NH). ^{13}C NMR (δ, ppm): 20.2 (CH_3), 91.3 (CH_2), 113.5, 116.3, 118.2, 119.9, 120.0, 122.1, 125.4, 127.6, 131.4, 142.6, 144.2, 152.8, 148.1, 157.9, 163.4, 171.7 (Ar-C), 173.2 (CO). Anal.% Calcd for $C_{21}H_{14}BrN_3O_3$ (436.27): C, 57.82; H, 3.23; N, 9.63. Found: C, 57.91; H, 3.17; N, 9.75.

*(4) 7-(4-Bromophenyl)-2-methyl-5-(2-thienyl)pyrido[2,3-d]pyrimidine-4(3H)-one (**14**).* Yield: (2.0 g, 63%, ethanol); m.p.: 248–250°C; IR (cm^{-1}, KBr): 3270 (NH), 1652 (C=O). ^{1}H NMR (δ, ppm): 2.35 (s, 3H, CH_3), 6.68–7.77 (m, 8H, Ar-H); 8.25 (s, 1H, NH). ^{13}C NMR (δ, ppm): 21.0 (CH_3), 118.8, 120.5, 121.8, 122.9, 125.2, 127.4, 129.3, 132.2, 138.3, 142.2, 147.1, 157.7, 163.1, 172.9 (Ar-C), 170.2 (CO). Anal.% Calcd for $C_{18}H_{12}BrN_3OS$ (398.28): C, 54.28; H, 3.04; N, 10.55. Found: C, 54.17; H, 3.00; N, 10.61.

*(5) 2-Methyl-7-(4-methoxyphenyl)-5-(2-thienyl)pyrido[2,3-d]pyrimidine-4(3H)-one (**15**).* Yield: (2.2 g, 65%, ethanol); m.p.: 200–202°C; IR (cm^{-1}, KBr): 3259 (NH), 1653 (C=O). ^{1}H NMR (δ, ppm): 2.36 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3); 6.81–8.00 (m, 8H, Ar-H); 8.22 (s, 1H, NH). ^{13}C NMR (δ, ppm): 20.8 (CH_3), 56.1 (OCH_3), 91.9 (CH_2), 113.5, 116.6, 118.8, 120.41, 120.6, 122.7, 125.5, 127.3, 131.3, 142.2, 144.7, 148.4, 160.1, 157.3, 164.3, 173.3 (Ar-C), 170.2 (CO). Anal.% Calcd for $C_{19}H_{15}N_3O_2S$ (349.41): C, 65.31; H, 4.33; N, 12.03. Found: C, 65.25; H, 4.29; N, 12.13.

*2.1.4. 4-Imino-3-phenyl-5,7-disubstituted-dihydropyrido[2,3-d]pyrimidine-2(1H)-thiones (**16**–**20**).* To a solution of the appropriate nicotinonitriles **1**–**5** (10 mmol) in pyridine (15 mL), phenyl isothiocyanate (0.15 g, 15 mmol) was added, and the mixture was refluxed for 4 h. After cooling, the solid product thus formed was filtered, washed thoroughly with water, dried, and recrystallized from the appropriate solvent.

*(1) 5-(Benzo[d][1,3]dioxol-5-yl)-4-imino-3,7-diphenyl-3,4-dihydropyrido[2,3-d]pyrimidine-2(1H)-thione (**16**).* Yield: (3.2 g, 82%, methanol); m.p.: 178–180°C; IR (cm^{-1}, KBr): 3328 (NH), 1635 (C=N), 1204 (C=S). ^{1}H NMR (δ, ppm): 6.12 (s, 2H, CH_2), 6.75 (s, 1H, C=NH); 6.78–7.99 (m, 15H, 14 Ar-H + NH). ^{13}C NMR (δ, ppm): 90.9 (CH_2), 108.1, 109.8, 113.5, 115.6, 120.2, 121.3, 124.5, 125.3, 127.1, 127.5, 128.8, 129.0, 131.4, 139.5, 139.7, 148.3, 149.0, 160.0 (Ar-C), 163.6 (C=NH), 179.8 (CS). Anal.% Calcd for $C_{26}H_{18}N_4O_2S$ (450.52): C, 69.32; H, 4.03; N, 12.44. Found: C, 69.24; H, 3.99; N, 12.40.

*(2) 4-Imino-3,7-diphenyl-5-(2-thienyl)-3,4-dihydropyrido[2,3-d]pyrimidine-2(1H)-thione (**17**).* Yield: (3.89 g, 82%, methanol); m.p.: 168–170°C; IR (cm^{-1}, KBr): 3287 (NH), 1639 (C=N), 1198 (C=S). ^{1}H NMR (δ, ppm): 6.86 (s, 1H, C=NH); 6.78–7.80 (m, 15H, 14 Ar-H + NH). ^{13}C NMR (δ, ppm): 109.2, 110.6, 121.5, 122.8, 124.3, 125.9, 127.1, 127.3, 127.6, 129.1, 132.3, 139.9, 142.4, 144.9, 149.7, 158.1, 162.1 (Ar-C), 164.7 (C=NH), 180.3 (C=S). Anal.% Calcd for $C_{23}H_{16}N_4S_2$ (412.53): C, 66.97; H, 3.91; N, 13.58. Found: C, 66.90; H, 3.88; N, 13.65.

*(3) 5-(Benzo[d][1,3]dioxol-5-yl)-7-(4-bromophenyl)-4-imino-3-phenyl-3,4-dihydropyrido[2,3-d]pyrimidine-2(1H)-thione (**18**).* Yield: (3.3 g, 78%, ethanol); m.p.: 198–200°C; IR (cm^{-1}, KBr): 3321 (NH), 1637 (C=N), 1203 (C=S). ^{1}H NMR (δ, ppm): 5.98 (s, 2H, CH_2), 6.78 (s, 1H, C=NH); 6.88–8.12 (m, 14H, 13 Ar-H + NH). ^{13}C NMR (δ, ppm): 91.3 (CH_2), 108.6, 109.8, 113.8, 116.1, 120.4, 124.8, 125.5, 127.1, 127.4, 128.8, 129.3, 131.6, 139.5, 140.0, 148.1, 149.2, 158.2, 160.8 (Ar-C), 164.4 (C=NH), 181.2 (C=S). MS: *m/z* 530 (M^+, 74), 529 (100), 449 (80), 369 (29), 361 (17), 330 (25), 132 (26), 84 (30), 75 (22). Anal.% Calcd for $C_{26}H_{17}BrN_4O_2S$ (529.41): C, 58.99; H, 3.24; N, 10.58. Found: C, 58.91; H, 3.26; N, 10.45.

*(4) 7-(4-Bromophenyl)-4-imino-3-phenyl-5-(2-thienyl)-3,4-dihydropyrido[2,3-d]pyrimidine-2(1H)-thione (**19**).* Yield: (3.4 g, 76%, ethanol); m.p.: 180–182°C; IR (cm^{-1}, KBr): 3284 (NH), 1642 (C=N), 1195 (C=S). ^{1}H NMR (δ, ppm): 6.81 (s, 1H, C=NH); 7.34–7.82 (m, 14H, 13 Ar-H + NH). ^{13}C NMR (δ, ppm): 108.4, 110.9, 121.6, 122.8, 124.5, 125.5, 126.8, 127.2, 127.4, 128.9, 132.4, 139.8, 142.5, 144.4, 149.0, 158.3, 160.0 (Ar-C), 164.6 (C=NH), 179.3 (C=S). Anal.% Calcd for $C_{23}H_{15}BrN_4S_2$ (491.43): C, 56.21; H, 3.08; N, 11.40. Found: C, 56.12; H, 3.06; N, 11.51.

*(5) 4-Imino-7-(4-methoxyphenyl)-3-phenyl-5-(2-thienyl)-3,4-dihydropyrido[2,3-d]pyrimidine-2(1H)-thione (**20**).* Yield: (2.3 g, 73%, DMF); m.p.: 190–192°C; IR (cm^{-1}, KBr): 3312 (NH), 1639 (C=N), 1188 (C=S). ^{1}H NMR (δ, ppm): 3.73 (s, 3H, OCH_3), 6.83 (s, 1H, C=NH); 6.79–7.94 (m, 13H, 12 Ar-H + NH). ^{13}C NMR (δ, ppm): 56.0 (OCH_3), 106.1, 114.6, 120.2, 121.6, 122.8, 124.5, 125.5, 126.8, 127.2, 127.4, 128.9, 132.4, 142.5, 144.4, 149.0, 158.3, 160.0 (Ar-C), 164.8 (C=NH), 179.6 (C=S). Anal.% Calcd for $C_{24}H_{18}N_4OS_2$ (422.56): C, 65.14; H, 4.10; N, 12.66. Found: C, 65.09; H, 4.17; N, 12.56.

*2.1.5. N-((3-Cyano-4,6-disubstituted-pyridin-2-yl)thiocarbamoyl)benzamides (**21**–**23**).* To a solution of the appropriate nicotinonitriles **1**–**5** (20 mmol) in dry acetone (30 mL), a solution of benzoyl isothiocyanate (1.65 g, 20 mmol) in dry acetone (10 mL) was added. The resulting mixture was heated under reflux for 10 h. The reaction mixture was concentrated and left overnight at room temperature. The separated crystalline product was filtered, washed with Et_2O, and recrystallized from the appropriate solvent.

*(1) N-((3-Cyano-6-phenyl-4-(2-thienyl)pyridin-2-yl)carbamothioyl)benzamide (**21**).* Yield: (3.7 g, 85%, methanol); m.p.: 126–128°C; IR (cm^{-1}, KBr): 3433 (NH), 2215 (CN), 1661 (C=O), 1166 (C=S). ^{1}H NMR (δ, ppm): 6.39 (s, 1H, CSNH); 7.10–8.14 (m, 14H, Ar-H); 8.43 (s, 1H, CONH). ^{13}C NMR (δ, ppm): 96.4, 111.2, 118.0 (CN), 122.4, 125.5, 126.3, 127.2, 127.4, 128.7, 129.0, 132.0, 133.3, 139.9, 142.3, 151.7, 161.8, 165.2 (Ar-C), 169.2 (CO), 180.1 (CS). MS: *m/z* 440 (M^+, 100), 414 (76), 333 (42), 311 (19), 252 (31), 206 (14), 105 (66), 83 (18), 75 (27). Anal.% Calcd for $C_{24}H_{16}N_4OS_2$ (440.54): C, 65.43; H, 3.66; N, 12.72. Found: C, 65.23; H, 3.96; N, 12.99.

*(2) N-((4-(Benzo[d][1,3]dioxol-5-yl)-6-(4-bromophenyl)-3-cyanopyridin-2-yl)carbamothioyl)benzamide (**22**).* Yield: (4.5 g, 82%, acetic acid); m.p.: 198–200°C; IR (cm^{-1}, KBr): 3425 (NH), 2226 (CN), 1665 (C=O), 1174 (C=S). ^{1}H NMR (δ, ppm,): 6.02 (s, 2H, CH_2), 6.35 (s, 1H, CSNH); 6.63–7.89 (m, 13H, Ar-H); 8.48 (s, 1H, CONH). ^{13}C NMR (δ, ppm): 91.7 (CH_2), 95.4, 113.0, 115.5, 117.7 (CN), 120.2, 122.3, 125.5, 126.6, 127.1, 127.3, 128.8, 129.2, 131.6, 133.4, 139.9, 142.4, 148.0, 151.7, 162.7, 165.2 (Ar-C), 168.1 (CO), 179.9 (CS). Anal.% Calcd for $C_{27}H_{17}BrN_4O_3S$ (557.42): C, 58.18; H, 3.07; N, 10.05. Found: C, 58.11; H, 3.09; N, 10.15.

*(3) N-((6-(4-Bromophenyl)-3-cyano-4-(2-thienyl)pyridin-2-yl)carbamothioyl)benzamide (**23**).* Yield: (4.1 g, 79%, acetic acid); m.p.: 210–212°C; IR (cm^{-1}, KBr): 3422 (NH), 2218 (CN), 1662 (C=O), 1170 (C=S). ^{1}H NMR (δ, ppm): 6.28 (s, 1H, CSNH); 7.02–8.32 (m, 12H, Ar-H); 8.52 (s, 1H, CONH). ^{13}C NMR (δ, ppm): 94.4, 111.0, 118.1 (CN), 122.8, 125.3, 126.5, 127.3, 127.5, 128.6, 129.1, 131.9, 133.5, 139.8, 142.6, 151.5, 161.9, 165.4 (Ar-C), 169.3 (CO), 180.4 (CS). Anal.% Calcd for $C_{24}H_{15}BrN_4OS_2$ (519.44): C, 55.50; H, 2.91; N, 10.79. Found: C, 55.14; H, 3.19; N, 11.01.

*2.1.6. 4-Amino-5,7-disubstituted-pyrido[2,3-d]pyrimidine-2(1H)-ones (**24**–**27**).* A mixture of the appropriate nicotinonitriles **1**–**5** (10 mmol) and urea (0.6 g, 10 mmol) was fused at 260–300°C using a sand bath for 1 h. The reaction mixture was allowed to attain room temperature; the crude solid product was treated with water, then rubbed with ethanol, filtered, and recrystallized from DMF/H_2O.

*(1) 4-Amino-5-(benzo[d][1,3]dioxol-5-yl)-7-phenylpyrido[2,3-d]pyrimidine-2(1H)-one (**24**).* Yield: (2.4 g, 71%); m.p.: 278–280°C; IR (cm^{-1}, KBr): 3280, 3323, 3435 (NH & NH_2), 1660 (C=O). ^{1}H NMR (δ, ppm): 5.98 (s, 2H, CH_2), 6.52 (s, 2H, NH_2), 6.74–8.13 (m, 10H, 9 Ar-H + NH). ^{13}C NMR

(δ, ppm): 93.5 (CH_2), 113.7, 115.8, 120.6, 124.4, 126.5, 127.2, 128.8, 129.0, 129.3, 139.8, 142.4, 148.1, 150.8, 158.5, 160.4, 164.4 (Ar-C), 166.2 (CO). Anal.% Calcd for $C_{20}H_{14}N_4O_3$ (358.36): C, 67.03; H, 3.94; N, 15.63. Found: C, 67.10; H, 3.89; N, 15.49.

*(2) 4-Amino-7-phenyl-5-(2-thienyl)pyrido[2,3-d]pyrimidine-2(1H)-one (**25**).* Yield: (2.1 g, 75%); m.p.: 280–282°C; IR (cm^{-1}, KBr): 3276, 3336, 34659 (NH & NH_2), 1665 (C=O). ^{1}H NMR (δ, ppm): 6.44 (s, 2H, NH_2), 6.82–8.15 (m, 10H, 9 Ar-H + NH). ^{13}C NMR (δ, ppm): 109.6, 110.0, 122.2, 125.5, 126.5, 127.1, 128.1, 129.4, 139.7, 142.3, 151.2, 159.1, 162.5, 165.1 (Ar-C), 166.4 (CO). Anal.% Calcd for $C_{17}H_{12}N_4OS$ (320.37): C, 63.73; H, 3.78; N, 17.49. Found: C, 63.81; H, 3.67; N, 17.57.

*(3) 4-Amino-5-(benzo[d][1,3]dioxol-5-yl)-7-(4-bromophenyl)pyrido[2,3-d]pyrimidine-2(1H)-one (**26**).* Yield: (2.2 g, 75%); m.p.: 260–262°C; IR (cm^{-1}, KBr): 3271, 3326, 3460 (NH & NH_2), 1667 (C=O). ^{1}H NMR (δ, ppm): 6.10 (s, 2H, CH_2), 6.48 (s, 2H, NH_2), 6.84–8.13 (m, 9H, 8 Ar-H + NH). ^{13}C NMR (δ, ppm): 92.6 (CH_2), 113.5, 115.6, 122.1, 125.5, 126.4, 127.2, 128.8, 129.0, 129.4, 139.6, 142.6, 148.1, 150.9, 157.8, 160.2, 164.8 (Ar-C). 166.2 (CO). Anal.% Calcd for $C_{20}H_{13}BrN_4O_3$ (437.25): C, 54.94; H, 3.00; N, 12.81. Found: C, 54.91; H, 2.97; N, 12.89.

*(4) 4-Amino-7-(4-bromophenyl)-5-(2-thienyl)pyrido[2,3-d]pyrimidine-2(1H)-one (**27**).* Yield: (2.1 g, 73%); m.p.: 250–252°C; IR (cm^{-1}, KBr): 3284, 3346, 3455 (NH & NH_2), 1664 (C=O). ^{1}H NMR (δ, ppm): 6.41 (s, 2H, NH_2); 6.97–8.01 (m, 8H, Ar-H); 8.02 (s, 1H, NH). ^{13}C NMR (δ, ppm): 108.1, 110.3, 122.7, 125.2, 126.7, 127.3, 127.4, 129.0, 139.3, 142.6, 144.5, 158.4, 159.3, 166.1 (Ar-C), 162.3 (CO). Anal.% Calcd for $C_{17}H_{11}BrN_4OS$ (399.27): C, 51.14; H, 2.78; N, 14.03. Found: C, 51.05; H, 2.74; N, 14.07.

*2.1.7. 4-Amino-5,7-disubstituted-pyrido[2,3-d]pyrimidine-2(1H)-thiones (**28**–**32**).* A mixture of the appropriate nicotinonitriles **1**–**5** (10 mmol) and thiourea (0.8 g, 10 mmol) was fused at 260–300°C using a sand bath for 1.5 h. The reaction mixture was worked up as described under compounds **24**–**27** and recrystallized from the appropriate solvent.

*(1) 4-Amino-5-(benzo[d][1,3]dioxol-5-yl)-7-phenylpyrido[2,3-d]pyrimidine-2(1H)-thione (**28**).* Yield: (2.1 g, 67%, ethanol); m.p.: 220–222°C; IR (cm^{-1}, KBr): 3272, 3345, 3462 (NH & NH_2), 1179 (C=S). ^{1}H NMR (δ, ppm): 5.98 (s, 2H, CH_2), 6.52 (s, 2H, NH_2), 6.74–8.13 (m, 10H, 9 Ar-H + NH). ^{13}C NMR (δ, ppm): 91.4 (CH_2), 113.4, 115.6, 120.3, 124.8, 126.6, 127.1, 128.4, 129.0, 129.5, 139.9, 142.5, 148.1, 150.6, 158.3, 160.5, 164.7 (Ar-C), 182.4 (CS). Anal.% Calcd for $C_{20}H_{14}N_4O_2S$ (374.42): C, 64.16; H, 3.77; N, 14.96. Found: C, 64.09; H, 3.76; N, 14.90.

*(2) 4-Amino-7-phenyl-5-(2-thienyl)pyrido[2,3-d]pyrimidine-2(1H)-thione (**29**).* Yield: (2.2 g, 52%, methanol); m.p.: 198–200°C; IR (cm^{-1}, KBr): 3277, 3325, 3438 (NH & NH_2), 1166 (C=S). ^{1}H NMR (δ, ppm): 6.44 (s, 2H, NH_2), 6.82–8.15 (m, 10H, 9 Ar-H + NH). ^{13}C NMR (δ, ppm): 108.6, 110.0, 122.2, 125.5, 126.8, 127.1, 128.3, 129.6, 139.7, 142.6, 150.7, 158.2, 162.2, 165.4 (Ar-C), 182.3 (CS). MS: *m/z* 336 (M^+, 74), 320 (100), 303 (72), 220 (31), 132 (15), 83 (11), 75 (25). Anal.% Calcd for $C_{17}H_{12}N_4S_2$ (336.43): C, 60.69; H, 3.60; N, 16.65. Found: C, 60.62; H, 3.57; N, 16.78.

*(3) 4-Amino-5-(benzo[d][1,3]dioxol-5-yl)-7-(4-bromophenyl)pyrido[2,3-d]pyrimidine-2(1H)-thione (**30**).* Yield: (2.6 g, 73%, ethanol); m.p.: 230–232°C; IR (cm^{-1}, KBr): 3288, 3346, 3438 (NH & NH_2), 1173 (C=S). ^{1}H NMR (δ, ppm): 5.96 (s, 2H, CH_2), 6.59 (s, 2H, NH_2), 6.84–8.03 (m, 9H, 8 Ar-H + NH). ^{13}C NMR (δ, ppm): 92.1 (CH_2), 113.5, 115.4, 120.1, 124.6, 126.4, 127.1, 128.8, 129.1, 129.5, 139.7, 142.7, 148.0, 151.0, 159.3, 160.5, 164.9 (Ar-C), 181.4 (CS). Anal.% Calcd for $C_{20}H_{13}BrN_4O_2S$ (453.31): C, 52.99; H, 2.89; N, 12.36. Found: C, 52.93; H, 2.87; N, 12.47.

*(4) 4-Amino-7-(4-bromophenyl)-5-(2-thienyl)pyrido[2,3-d]pyrimidine-2(1H)-thione (**31**).* Yield: (1.7 g, 52%, ethanol); m.p.: 243–245°C; IR (cm^{-1}, KBr): 3279, 3329, 3460 (NH & NH_2), 1177 (C=S). ^{1}H NMR (δ, ppm): 6.49 (s, 2H, NH_2), 6.67–8.11 (m, 9H, 8 Ar-H + NH). ^{13}C NMR (δ, ppm): 109.3, 110.7, 122.1, 125.5, 127.1, 127.6, 127.9, 129.4, 139.6, 143.2, 150.7, 158.1, 162.0, 165.5 (Ar-C), 182.7 (CS). Anal.% Calcd for $C_{17}H_{11}BrN_4S_2$ (415.33): C, 49.16; H, 2.67; N, 13.49. Found: C, 49.12; H, 2.64; N, 13.38.

*(5) 4-Amino-7-(4-methoxyphenyl)-5-(2-thienyl)pyrido[2,3-d]pyrimidine-2(1H)-thione (**32**).* Yield: (2.6 g, 71%, ethanol); m.p.: 298–300°C; IR (cm^{-1}, KBr): 3277, 3344, 3431 (NH & NH_2), 1168 (C=S). ^{1}H NMR (δ, ppm): 2.89 (s, 3H, OCH_3), 6.75 (s, 2H, NH_2); 6.94–8.27 (m, 9H, 8 Ar-H + NH). ^{13}C NMR (δ, ppm): 56.1 (OCH_3) 108.8, 110.6, 114.6, 122.9, 125.3, 127.1, 128.2, 129.0, 139.7, 142.2, 144.6, 157.8, 158.7, 164.5 (Ar-C), 183.3 (CS). Anal.% Calcd for $C_{18}H_{14}N_4OS_2$ (366.46): C, 59.00; H, 3.85; N, 15.29. Found: C, 58.86; H, 3.83; N, 15.17.

*2.1.8. 2-Alkylthio-4-amino-5,7-disubstituted-pyrido[2,3-d]pyrimidines (**33**–**37**).* To a stirred solution of proper thiones **30**–**32** (10 mmol) in 1N NaOH (5 mL) and ethanol (2 mL), the selected alkyl iodide (12 mmol) was added. The reaction mixture was stirred at room temperature for 2-3 h and the precipitated product was filtered, washed with cold ethanol, dried, and recrystallized from proper solvent.

*(1) 4-Amino-5-(benzo[d][1,3]dioxol-5-yl)-7-(4-bromophenyl)-2-(methylthio)pyrido[2,3-d]pyrimidine (**33**).* Yield: (3.5 g, 76%, methanol); m.p. 240–242°C; IR (cm^{-1}, KBr): 3365, 3435 (NH_2). ^{1}H NMR (δ, ppm): 2.49 (s, 3H, SCH_3); 6.41 (s, 2H, CH_2); 6.92–8.06 (m, 10H, 8 Ar-H + NH_2). ^{13}C NMR (δ, ppm): 19.4 (SCH_3), 91.4 (CH_2), 102.1, 113.8, 115.7, 120.1, 120.2, 121.7, 127.4, 129.1, 131.2, 132.1, 138.9, 148.1, 149.3, 157.8, 158.2, 167.1, 167.7 (Ar-C). Anal.% Calcd for $C_{21}H_{15}BrN_4O_2S$ (467.34): C, 53.97; H, 3.24; N, 11.99. Found: C, 53.87; H, 3.19; N, 12.14.

*(2) 4-Amino-5-(benzo[d][1,3]dioxol-5-yl)-7-(4-bromophenyl)-2-(ethylthio)pyrido[2,3-d]pyrimidine (**34**).* Yield: (2.3 g, 59%, ethanol); m.p.: 176–178°C; IR (cm^{-1}, KBr): 3288, 3429

(NH_2). ^{1}H NMR (δ, ppm): 1.24 (t, J = 6 Hz, 3H, CH_3); 2.88 (q, J = 6 Hz, SCH_2), 6.41 (s, 2H, CH_2); 6.92–8.06 (m, 10H, 8 Ar-H + NH_2). ^{13}C NMR (δ, ppm): 16.1 (CH_3), 28.1 (SCH_2), 91.4 (CH_2), 102.1, 113.8, 115.7, 120.1, 120.2, 121.7, 127.4, 129.1, 131.2, 132.1, 138.9, 148.1, 149.3, 157.8, 158.2, 167.1, 167.7 (Ar-C). Anal.% Calcd for $C_{22}H_{17}BrN_4O_2S$ (481.36): C, 54.89; H, 3.56; N, 11.64. Found: C, 54.79; H, 3.53; N, 11.72.

*(3) 4-Amino-7-(4-bromophenyl)-2-(methylthio)-5-(2-thienyl)pyrido[2,3-d]pyrimidine (**35**).* Yield: (2.3 g, 66%, ethanol); m.p.: 185–187°C; IR (cm^{-1}, KBr): 3391, 3452 (NH_2). ^{1}H NMR (δ, ppm): 2.42 (s, 3H, SCH_3); 6.55 (s, 2H, NH_2) 7.10–7.89 (m, 8H, Ar-H). ^{13}C NMR (δ, ppm): 19.1 (SCH_3), 100.6, 120.9, 121.7, 122.8, 125.3, 127.4, 129.2, 129.5, 132.4, 138.6, 142.7, 145.0, 159.6, 158.7, 167.1, 167.6 (Ar-C). Anal.% Calcd for $C_{18}H_{13}BrN_4S_2$ (429.36): C, 50.35; H, 3.05; N, 13.05. Found: C, 50.41; H, 3.00; N, 13.18.

*(4) 4-Amino-7-(4-methoxyphenyl)-2-(methylthio)-5-(2-thienyl)pyrido[2,3-d]pyrimidine (**36**).* Yield: (2.6 g, 61%, DMF/H_2O); m.p.: 238–240°C; IR (cm^{-1}, KBr): 3385, 3447 (NH_2). ^{1}H NMR (δ, ppm): 2.45 (s, 3H, SCH_3), 3.82 (s, 3H, OCH_3), 6.62 (s, 2H, NH_2); 6.74–8.12 (m, 8H, Ar-H). ^{13}C NMR (δ, ppm): 56.1 (OCH_3), 108.7, 110.4, 114.4, 122.3, 125.5, 127.1, 128.3, 129.2, 139.6, 142.2, 144.8, 157.8, 158.3, 167.3, 167.8 (Ar-C). MS: m/z 380 (M^+, 100), 364 (70), 333 (82), 317 (54), 286 (22), 234 (30), 132 (26), 83 (16), 75 (24). Anal.% Calcd for $C_{19}H_{16}N_4OS_2$ (380.49): C, 59.98; H, 4.24; N, 14.73. Found: C, 59.91; H, 4.20; N, 14.84.

*(5) 4-Amino-2-(ethylthio)-7-(4-methoxyphenyl)-5-(2-thienyl)pyrido[2,3-d]pyrimidine (**37**).* Yield: (2.1 g, 61%, DMF/H_2O); m.p.: 238–240°C; IR (cm^{-1}, KBr): 3378, 3425 (NH_2). ^{1}H NMR (δ, ppm): 1.28 (t, J = 6 Hz, 3H, CH_3); 2.85 (q, J = 6 Hz, 2H, SCH_2), 3.81 (s, 3H, OCH_3), 6.60 (s, 2H, NH_2); 6.75–8.04 (m, 8H, Ar-H). ^{13}C NMR (δ, ppm): 15.7 (CH_3), 28.0 (SCH_2), 56.0 (OCH_3), 108.4, 110.1, 114.5, 122.2, 125.5, 127.0, 128.2, 129.1, 139.9, 142.5, 144.7, 157.7, 158.2, 167.0, 167.6 (Ar-C). Anal.% Calcd for $C_{20}H_{18}N_4OS_2$ (394.51): C, 60.89; H, 4.60; N, 14.20. Found: C, 60.79; H, 4.57; N, 14.34.

2.2. Biological Activity

2.2.1. In Vitro MTT Cytotoxicity Assay. The synthesized compounds were investigated for their *in vitro* cytotoxic effect via the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [25, 26] against a panel of three human tumor cell lines, namely, Caucasian breast adenocarcinoma MCF7, hepatocellular carcinoma HepG2, and colon carcinoma HT29 and a normal nontransformed human foreskin fibroblast Hs27 cell line. The procedures were done in a sterile area using a laminar flow cabinet biosafety class II level (Baker, SG403INT, Stanford, ME, USA). Cells were batch-cultured for 10 days and then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37°C for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added, and cells were incubated either alone (negative control) or with different concentrations of the test compounds to give a final concentration of 100-50-25-12.5-6.25-3.125-1.56-0.78 μg/mL. DMSO was employed as a vehicle for dissolution of the tested compounds and its final concentration on the cells was less than 0.2%. Cells were suspended in RPMI 1640 medium (for HepG2 and HT29 cell lines) and DMEM (for MCF 7 cell line), 1% antibiotic-antimycotic mixture (10,000 IU/mL Penicillin Potassium, 10,000 μg/mL Streptomycin Sulphate, and 25 μg/mL Amphotericin B), and 1% L-Glutamine in 96-well flat bottom microplate at 37°C under 5% CO_2. After 24 h of incubation, the medium was aspirated and 40 μL of MTT salt (2.5 μg/mL) was added to each well and incubated for further 4 h at 37°C under 5% CO_2. To stop the reaction and dissolve the formed crystals, 200 μL of 10% sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37°C. The absorbance was then measured using a microplate multiwell reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. The results are presented in Table 1 as LC_{50} (μM) which is the lethal concentration of the compound which causes death of 50% of the cells in 24 h.

2.2.2. In Vitro Antibacterial and Antifungal Activities. All of the newly synthesized compounds were evaluated for their *in vitro* antibacterial activity against *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), and *Micrococcus luteus* (ATCC 21881) as examples of Gram-positive bacteria and *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumonia* (clinical isolate) as examples of Gram-negative bacteria. They were also evaluated for their *in vitro* antifungal potential against *Candida albicans* (ATCC 10231) and *Aspergillus niger* (recultured) fungal strains. Each 100 mL of sterile molten agar (at 45°C) received 1 mL of 6 h-broth culture and then the seeded agar was poured into sterile Petri dishes. Cups (8 mm in diameter) were cut in the agar. Each cup received 0.1 mL of the 1 mg/mL solution of the test compounds. The plates were then incubated at 37°C for 24 h or, in case of *C. albicans,* for 48 h. A control using DMSO without the test compound was included for each organism. Ampicillin trihydrate and Clotrimazole were used as reference drugs. The results were recorded for each tested compound as the average diameter of inhibition zones (IZ) of bacterial or fungal growth around the discs in mm. The minimal inhibitory concentrations (MIC) of the most active compounds were measured using the twofold serial broth dilution method [27]. The test organisms were grown in their suitable broth: 24 h for bacteria and 48 h for fungi at 37°C. Twofold serial dilutions of solutions of the test compounds were prepared [28] using 200, 100, 50, 25, 12.5, and 6.25 μg/mL. The tubes were then inoculated with the test organisms; each 5 mL received 0.1 mL of the above inoculum and was incubated at 37°C for 48 h. Then, the tubes were observed for the presence or absence of microbial growth.

Table 1: Cytotoxic effects LC_{50} (μM)[a] of the active compounds on three human tumor and nontransformed cell lines and using the MTT assay.

Compound number	Human tumor cell lines			Nontransformed normal cell line
	Colon carcinoma (HT29)	Hepatocellular carcinoma (HePG2)	Breast cancer (MCF 7)	Foreskin fibroblast (Hs27)
21	70.5	—[b]	—	NT[c]
22	46.7	98.9	97.3	NT
23	62.2	—	—	NT
30	113.6	107	—	NT
32	153.4	111.3	123.0	NT
33	25.2	64.6	6.4	>200
34	28.8	70.1	7.9	>200
36	40.4	97.7	22.8	>200
37	26.9	71.2	8.91	>200
Doxorubicin[d]	40.0	3.0	4.0	NT

[a]LC_{50}: lethal concentration of the compound which causes death of 50% of cells in 24 h; [b]totally inactive against this cell line; [c]NT: not tested; [d]**Doxorubicin**: positive control cytotoxic agent.

3. Results and Discussion

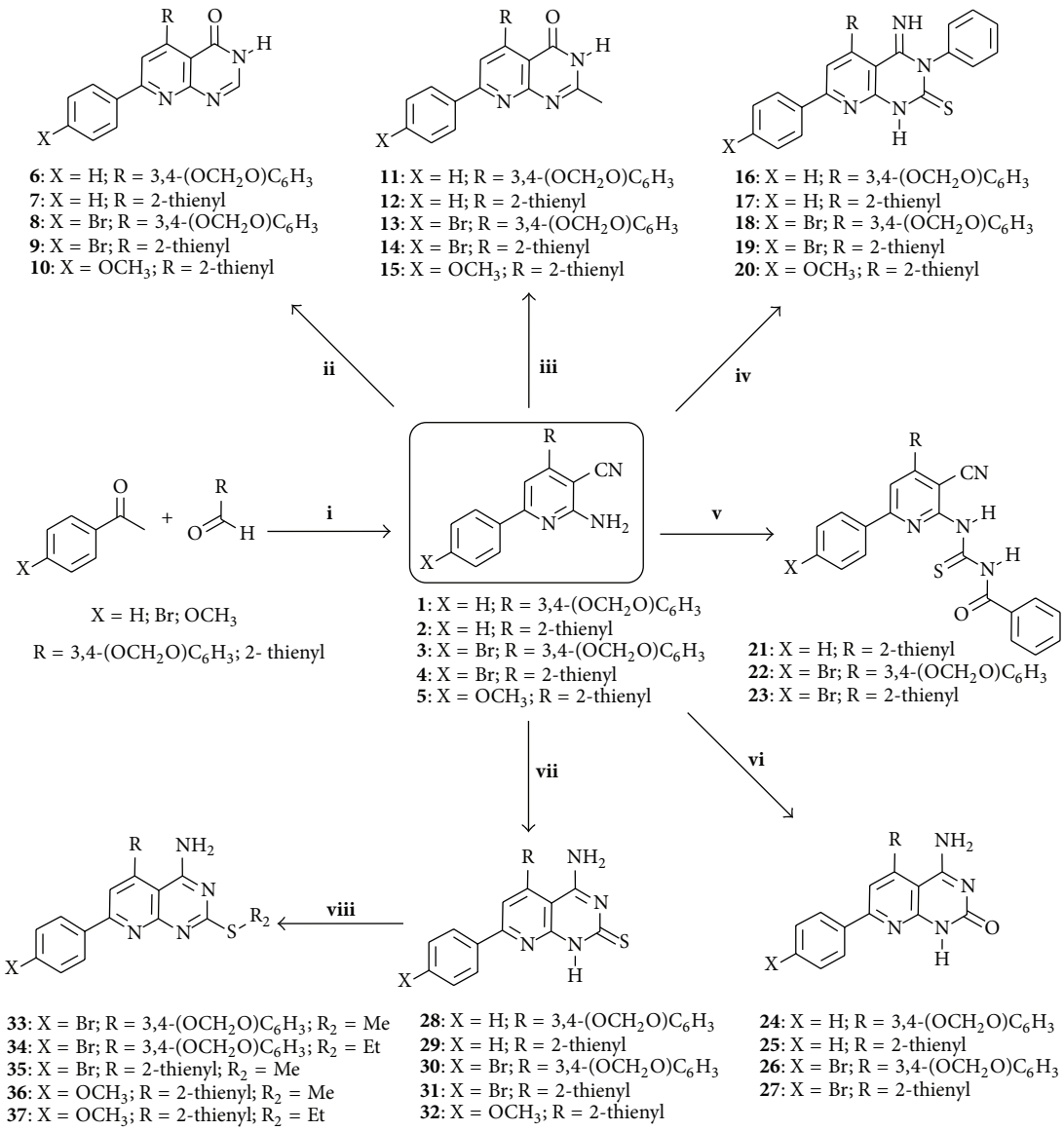
3.1. Chemistry. The pathways adopted for the preparation of the intermediate and target compounds are illustrated in Scheme 1. Preparation of the key intermediates 2-amino-4,6-disubstituted nicotinonitriles **1–5** was performed according to Hantzsch-type synthesis via multicomponent one-pot cyclocondensation of the appropriate aromatic aldehyde (piperonal or thiophene-2-carbaldehyde) with the substituted acetophenone, malononitrile, and ammonium acetate as a nitrogen source. The reaction passes through a two-step procedure that involved the formation of chalcones via Claisen-Schmidt condensation, followed by cyclocondensation with malononitrile and ammonium acetate. When compounds **1–5** were heated with either formic acid or acetic anhydride, the targeted 5,7-disubstituted pyrido[2,3-*d*]pyrimidine-4(3*H*)-ones **6–10** and their 2-methyl analogs **11–15,** respectively, were successfully obtained. The IR spectra of the latter compounds showed the absence of the CN group absorption and the appearance of new sharp absorption bands at 1656–1648 cm^{-1} attributed to the newly formed C=O groups at position 4, beside the NH absorption bands at 3320–3259 cm^{-1}. Meanwhile, the ^{1}H-NMR spectra of compounds **11–15** showed new singlets at δ 2.28–2.36 ppm due to the newly introduced CH_3 group, whereas their ^{13}C NMR spectral data exhibited new singlets at δ 20.2–21.1 ppm due to the new CH_3 group and the CO signals at δ 168.8–173.2 ppm.

Reacting compounds **1–5** with the phenyl isothiocyanate in pyridine medium led to the formation of the substituted dihydropyrido[2,3-*d*]pyrimidine-2(1*H*)-thiones **16–20**. Their IR spectra showed new bands at 1642–1635 cm^{-1} and at 1204–1188 cm^{-1} corresponding to the C=N moiety and to the C=S group, respectively. Their ^{13}C NMR spectral data were characterized by the presence of two signals at δ 179.3–181.2 and δ 164.4–164.8 ppm for the C=S and C=NH groups, respectively. However, condensing the starting compounds **2–4** with benzoyl isothiocyanate in acetone afforded the corresponding thioureido derivatives **21–23**. Their IR spectra revealed the CN absorption bands at 2226–2215 cm^{-1}, in addition to the C=O and C=S bands at 1665–1661 and 1174–1166 cm^{-1}, respectively, whereas their ^{13}C NMR spectra exhibited two characteristic signals at δ 168.1–169.3 and 179.9–180.4 ppm for the CO and CS carbons, respectively.

Furthermore, direct condensation of the starting compounds **1–4** with urea at 260–300°C was attempted to produce the target 4-amino-5,7-disubstituted-pyrido[2,3-*d*]pyrimidine-2(1*H*)-ones **24–27**. The newly introduced C=O function was obvious both in the IR at 1667–1660 cm^{-1} (beside the lack of CN absorption) and in the ^{13}C NMR spectra at δ 166.1–166.4 ppm. Analogously, when compounds **1–5** were directly fused with thiourea at 260–300°C, the corresponding 4-amino-5,7-disubstituted-pyrido[2,3-*d*]pyrimidine-2(1*H*)-thiones **28–32** were obtained. Their ^{13}C NMR spectra clearly revealed the signals attributed to the C=S carbons at δ 181.4–183.3 ppm. Finally, the synthesis of the targeted alkylthio derivatives **33–37** was successfully achieved via thioalkylation of the 2-thione derivatives **30–32** with either methyl or ethyl iodide in 1N NaOH medium. The ^{1}H-NMR spectra of **33**, **35,** and **36** showed the methylthio singlets at δ 2.42–2.49 ppm, whereas the ethylthio derivatives **34** and **37** exhibited triplets at δ 1.24–1.28 ppm and quartets at δ 2.85–2.88 ppm due to the ethyl CH_3 and CH_2 protons, respectively.

3.2. Biological Evaluation

3.2.1. In Vitro MTT Cytotoxicity Assay. All the newly synthesized compounds were evaluated for their *in vitro* cytotoxic effect via the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [25, 26] against a panel of three human tumor cell lines, namely, colon carcinoma HT29, hepatocellular carcinoma HePG2, and Caucasian breast adenocarcinoma MCF7. The results are presented in Table 1 as LC_{50} (μM) which is the lethal concentration of the compound that causes death of 50% of the cells in 24 h.



Scheme 1: Synthesis of the target compounds **1**–**37**. Reagents and reaction conditions: **i**: malononitrile, NH_4OAc, EtOH, reflux, 4-5 h; **ii**: HCOOH, heating, 30 min.; **iii**: Ac_2O, heating, 10 min.; **iv**: PhNCS, pyridine, reflux, 4 h; **v**: PhCONCS, acetone, reflux, 10 h; **vi**: urea, 260–300°C, 1 h; **vii**: thiourea, 260–300°C, 1.5 h; **viii**: R_2X, 1N NaOH, ethanol, r.t., 2-3 h.

The obtained data revealed that the three tested human tumor cell lines exhibited variable degree of sensitivity profiles towards nine of the tested compounds, namely, **21**, **22**, **23**, **30**, **32**, **33**, **34**, **36**, and **37**, whereas the other twenty-six compounds were either marginally active or even totally inactive. Regarding the activity against the human colon carcinoma HT29, this cell line proved to be very sensitive to all the nine active compounds. In particular, it revealed distinctive sensitivity towards compounds **33**, **34**, and **37** (LC_{50} 25.2, 28.8, and 26.9 μM, resp.) even higher than doxorubicin (LC_{50} 40.0 μM), the reference standard cytotoxic agent utilized in this assay. Meanwhile, compounds **22** and **36** (LC_{50} 46.7 and 40.4 μM, resp.) were nearly equipotent to doxorubicin (LC_{50} 40.0 μM), whereas compounds **21** and **23** (LC_{50} 70.5 and 62.2 μM, resp.) showed moderate cytotoxic potential against the same cell line. Shifting to the hepatocellular carcinoma HepG2, this cell line showed mild to weak sensitivity towards seven of the tested analogs with LC_{50} range 64.6–111.3 μM, when compared to doxorubicin (LC_{50} 3.0 μM). Among these, the highest activity was displayed by compounds **33**, **34**, and **37** (LC_{50} 64.4, 70.1, and 71.2 μM, resp.). On the other hand, the human breast cancer MCF 7 emerged as the least sensitive among the cell lines tested as its growth was affected by the presence of only six test compounds. However, a remarkable growth inhibition potential was shown by analogs **33**, **34**, and **37** as evidenced from their LC_{50} values (LC_{50} 6.4, 7.9, and 8.91 μM, resp.), which represents about 40–60% of the activity of doxorubicin (LC_{50} 4.0 μM).

Further interpretation of the results revealed that compounds **33**, **34**, **36**, and **37** showed considerable broad spectrum cytotoxic activity against the three tested human tumor cell lines. In particular, compounds **33**, **34**, and **37** proved to be the most active members in this study with special effectiveness against both the colon carcinoma HT29 (almost twice as active as doxorubicin; LC_{50} 25.2, 28.8, and 26.9 versus 40 μM, resp.) and human breast cancer MCF 7 (about 40–60% of the activity of doxorubicin; LC_{50} 6.4, 7.9, and 8.91 versus 4.0 μM, resp.).

A close examination of the structures of the active compounds showed that the nature of substituents (X and/or R), together with ring entity (mono- or bicyclic), seemed to influence the cytotoxic activity. In this context, compounds substituted with the 4-bromo- or 4-methoxyphenyl moities (X = Br and OCH_3) together with the benzo[*d*][1,3]dioxol-5-yl counterpart (**22**, **30**, **33**, and **34**) were in favour of better cytotoxic activity, when compared with their 2-thienyl congeners (**23**, **32**, **36**, and **37**), as revealed from their LC_{50} values in Table 1. Moreover, the bicyclic pyrido[2,3-*d*]pyrimidines proved to be more active than the monocyclic nicotinonitriles. In this view, although the starting nicotinonitriles **1**–**5** lacked cytotoxic efficacy, yet the thiocarbamoyl benzamide derivatives **21**–**23** showed overall mild to moderate activity, among which analog **22** (X = Br; R = 3,4-$(OCH_2O)C_6H_3$) was relatively the most active regarding both potency and spectrum. Cyclization of the nicotinonitriles **1**–**5** with different reagents yielded variably substituted bicyclic pyrido[2,3-*d*]pyrimidines **6**–**10**, **11**–**15**, **16**–**20**, and **24**–**27**, which were all inactive against the three tested cell lines. However, isosteric replacement of 2-one functionality in pyrido[2,3-*d*]pyrimidine-2(1*H*)-ones **24**–**27**, with a 2-thione group, yielded two weakly active analogs, namely, **30** (X = Br; R = 3,4-$(OCH_2O)C_6H_3$) and **32** (X = OCH_3; R = 2-thienyl). On the other hand, a remarkable improvement in both cytotoxic spectrum and potency was observed by thioalkylation of the 2-thione function of compounds **28**–**32** either with a methyl or ethyl group, where four potentially active compounds **33**, **34**, **36,** and **37** were obtained. Among these, the analogs compounds **33** (X = Br; R = 3,4-$(OCH_2O)C_6H_3$; $R_2 = CH_3$), **34** (X = Br; R = 3,4-$(OCH_2O)C_6H_3$; $R_2 = C_2H_5$), and **37** (X = OCH_3; R = 2-thienyl; $R_2 = C_2H_5$) stemmed as the most active members in this study.

3.2.2. In Vitro Differential Cytotoxicity Effect. Owing to the known common side effects often induced by chemotherapeutic agents exemplified by the possible destruction of normal cells, it was thought worthwhile to evaluate the *in vitro* differential cytotoxic effect of the most active analogs **33**, **34**, **36**, and **37** on the nontransformed human foreskin fibroblast Hs27 cell line, under the same previously mentioned MTT assay experimental conditions.

Considering the obvious selective inhibitory potential displayed by the analogs (**33**, **34**, **36**, and **37**) on the growth of the human breast MCF7 and colon HT29 cancer cell lines, the results revealed that the potential cytotoxic activity exhibited by **33**, **34**, and **37** (LC_{50} values range 6.4–28.8 μM) was confirmed by their clear differential cytotoxic behavior, showing a marginal inhibitory effect on the growth of the nontransformed human foreskin fibroblast Hs27 cell line (LC_{50} values > 200 μM), whereas a lower differential cytotoxic profile was shown by analog **36** against the tested normal cell line (LC_{50} > 200 μM), when compared with its LC_{50} values against the human breast MCF7 and colon HT29 cancer cell lines (22.8 and 40.4 μM, resp.) (Table 1).

3.2.3. In Vitro Antibacterial and Antifungal Activities. All compounds were evaluated for their *in vitro* antibacterial activity against *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), and *Micrococcus luteus* (ATCC 21881) as examples of Gram positive bacteria and *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumonia* (clinical isolate) as examples of Gram-negative bacteria. They were also evaluated for their *in vitro* antifungal potential against *Candida albicans* (ATCC 10231) and *Aspergillus niger* (recultured) fungal strains. Agar-diffusion method was used for determination of preliminary antibacterial and antifungal activity. Ampicillin trihydrate (antibiotic) and Clotrimazole (antifungal) were used as reference drugs [27]. Dimethylsulfoxide (DMSO) was used as a blank and showed no antimicrobial activity. The results were recorded for each tested compound as the average diameter of inhibition zones (IZ) of bacterial or fungal growth around the discs in mm. The minimum inhibitory concentration (MIC, μg/mL) was determined for compounds that showed significant growth inhibition zones (≥13 mm) using the twofold serial dilution method [29].

As revealed from MIC data recorded in Table 2, nineteen of the newly synthesized compounds displayed variable inhibitory effects on the growth of the tested Gram-positive and Gram-negative microorganisms with pronounced activity against *S. aureus* and *E. coli* bacterial strains. In addition, some members exhibited moderate antifungal activity against *C. albicans*, whereas all the tested compounds lacked antifungal activity against *Aspergillus niger.*

Among the tested Gram-positive bacterial strains, two organisms, namely, *S. aureus* and *B. subtilis,* showed relatively high sensitivity towards the tested compounds. Concerning the activity against Gram-positive bacteria, compound **33** (MIC 6.25 μg/mL) was equipotent to ampicillin against *S. aureus;* meanwhile analogs **30**, **34**, and **36** (MIC 12.5 μg/mL) and **18**, **20**, **35**, and **37** (MIC 25 μg/mL) showed 50% and 25% of the activity of ampicillin, respectively. With regard to the activity against *B. subtilis*, compounds **33** and **34** (MIC 25 μg/mL) exhibited half the activity of ampicillin (MIC 12.5 μg/mL). *M. luteus* was the least sensitive Gram-positive microorganism towards the majority of the tested compounds, except analog **33** which showed 50% of the activity of ampicillin (MIC 25 versus 12.5 μg/mL). On the other hand, investigation of anti-Gram-negative efficacy of the active compounds revealed that three analogs **30**, **33**, and **34** were able to produce a distinctive growth inhibitory profile against *E. coli* being equipotent to ampicillin (MIC 6.25 μg/mL), whereas compounds **31**, **36**, and **37** (MIC 12.5 μg/mL) were 50% less active than ampicillin against the same organism. Moreover, the tested *P. aeruginosa* strain showed high sensitivity towards compound **33** (MIC 12.5 μg/mL) which was equiactive with ampicillin, whereas the rest of the active

Table 2: Minimal inhibitory concentrations (MIC; μg/mL) of the tested compounds.

Compound number	*S. aureus*	*B. subtilis*	*M. luteus*	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*C. albicans*
3	50	50	—[a]	25	100	—	—
4	50	100	—	50	—	—	—
5	50	100	200	100	200	—	—
13	100	100	100	50	100	—	—
15	100	200	—	100	100	200	—
18	25	100	50	25	50	100	100
19	100	100	—	50	50	—	—
20	25	100	—	25	50	200	100
21	100	100	50	100	200	50	—
22	50	100	100	50	100	50	—
23	50	100	200	100	50	100	—
29	100	100	50	50	100	200	100
30	12.5	50	50	6.25	25	100	50
31	50	100	—	12.5	200	100	50
33	6.25	25	25	6.25	12.5	50	12.5
34	12.5	25	100	6.25	25	100	25
35	25	100	200	25	50	—	100
36	12.5	50	100	12.5	25	—	25
37	25	50	50	12.5	100	—	50
Ampicillin	6.25	12.5	12.5	6.25	12.5	12.5	—
Clotrimazole	—	—	—	—	—	—	6.25

[a]Inactive: MIC > 200 μg/mL.

compounds revealed moderate to weak activity against the same strain (MIC range 25–200 μg/mL). Shifting to the antifungal potential, ten compounds **18**, **20**, **29**–**31**, and **33**–**37** were able to display appreciable growth inhibitory activity against *C. albicans* (MIC values 12.5–100 μg/mL, resp.) when compared with Clotrimazole (MIC 6.25 μg/mL), the standard antifungal agent utilized in this assay. Among these, analog **33** exhibited the best antifungal activity (MIC 12.5 μg/mL) that represents 50% of the standard. It is to be noted that all the tested compounds lacked any antifungal activity against *Aspergillus niger*.

Structurally, the nineteen active compounds represent two variations: the substituted nicotinonitriles and the bicyclic pyrido[2,3-*d*]pyrimidines. In general, relatively better antibacterial potential and spectrum were mostly confined to the derivatives comprising the bromine (X = Br) and the benzo[*d*][1,3]dioxol-5-yl groups, when compared with their methoxylated (X = OCH_3) and 2-thienyl congeners. Besides, better antimicrobial activity was associated with the bicyclic pyrido[2,3-*d*]pyrimidines when compared with the monocyclic nicotinonitrile precursors (Table 2). In this context, while the key nicotinonitrile precursors **3**–**5** showed mild antimicrobial activity, yet condensation of the 2-amino group with benzoyl isothiocyanate afforded three thiocarbamoyl benzamide derivatives **21**–**23** which revealed a slight improvement in the antimicrobial spectrum although not in efficacy. Annelation of nicotinonitriles **1**–**5** into the pyrido[2,3-*d*]pyrimidine-4(3*H*)-ones **6**–**10** resulted in complete abolishment of activity, whereas introducing a methyl group at C_2 to the latter ring system furnished two 2-methyl-pyrido[2,3-*d*]pyrimidine-4(3*H*)-one derivatives **13** and **15** which were noticeably less active than their parent compounds **3** and **5**. Furthermore, reaction of starting nicotinonitriles **1**–**5** with phenyl isothiocyanate gave rise to three active compounds **18**–**20**, among which analog **18** (X =Br; R = 3,4-$(OCH_2O)C_6H_3$) showed relatively better antimicrobial spectrum and potential. Although cyclization of nicotinonitriles **1**–**5** with urea into the corresponding pyrido[2,3-*d*]pyrimidine-2(1*H*)-ones **24**–**27** led to total loss of activity, yet isosteric replacement of the 2-one with a 2-thione functionality yielded three significantly active broad spectrum compounds **29**–**31**, among which analog **30** (X = Br; R = 3,4-$(OCH_2O)C_6H_3$) was the most active being equipotent to ampicillin against *E. coli*, together with a mild antifungal activity. Finally, thioalkylation of the 2-thione function of **28**–**32** afforded five methyl- or ethylthio derivatives **33**–**37** with a great enhancement in both antimicrobial spectrum and potential. In particular, compound **33** (X = Br; R = 3,4-$(OCH_2O)C_6H_3$; $R_2 = CH_3$) proved to be equipotent to ampicillin against *S. aureus*, *E. coli,* and *P. aeruginosa,* together with a noticeable antifungal activity against *C. albicans,* whereas analog **34** (X = Br; R = 3,4-$(OCH_2O)C_6H_3$; $R_2 = C_2H_5$) was equiactive with ampicillin against *E. coli.*

4. Conclusions

Thirty-seven polysubstituted pyridines and some derived bicyclic pyrido[2,3-*d*]pyrimidine ring systems supported with various chemotherapeutically active functionalities were successfully synthesized, characterized, and evaluated for

their biological activity as cytotoxic and/or antimicrobial agents. Globally, the data obtained from both the cytotoxic and antimicrobial assays suggested that higher biological activities were confined mainly to compounds comprising the 4-bromo, 4-methoxy, benzo[*d*][1,3]dioxol-5-yl substituents, and the bicyclic pyrido[2,3-*d*]pyrimidines rather than the monocyclic nicotinonitriles.

Regarding the cytotoxic activity, nine compounds were able to show variable cytotoxic efficiency, among which alkylthio analogs **33**, **34**, and **37** proved to be the most active members with considerable broad spectrum cytotoxic activity against the three tested human tumor cell lines and particular effectiveness against both the colon carcinoma HT29 (almost twice as active as doxorubicin; LC_{50} 25.2, 28.8, and 26.9 versus 40 μM, resp.) and human breast cancer MCF 7 (about 40–60% of the activity of doxorubicin; LC_{50} 6.4, 7.9, and 8.91 versus 4.0 μM, resp.). In addition, the same three analogs showed a clear differential cytotoxic profile as they exhibited a marginal inhibitory effect on the growth of the normal nontransformed human foreskin fibroblast Hs27 cell line (LC_{50} values > 200 μM).

On the other hand, the results of the *in vitro* antimicrobial screening revealed that nineteen compounds were able to exhibit significant antibacterial potential against both Gram-positive and Gram-negative bacteria, together with mild to moderate antifungal activities. The pyrido[2,3-*d*]pyrimidine-2(1*H*)-thione **30** in connection with its alkylthio derivatives **33** and **34** stemmed as the most active antimicrobial members being equipotent to ampicillin against *S. aureus*, *E. coli,* and *P. aeruginosa,* together with a noticeable antifungal activity against *C. albicans*. Globally, the distinctive cytotoxic and antimicrobial behavior displayed by alkylthio derivatives **33** and **34** are concordant with several literature findings that emphasized the role of thioethers in enhancing the antimicrobial and antitumor activities [28, 30, 31]. This makes such type of compounds a fruitful matrix for further development of more potent and selective anticancer and/or antimicrobial agents. In particular, compounds **33** and **34** could be considered as possible dual antimicrobial-anticancer candidates that deserve further investigation and derivatization in order to explore the scope and limitation of their biological activities.

Competing Interests

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

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