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ARTICLE

Synthesis of various fused heterocyclic rings from thiazolopyridine and their pharmacological and antimicrobial evaluations

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Abstract

Various fused-heterocyclic-derivatives containing thiazolopyridine moieties has been synthesized by allowing 5-aminothiazolo[3,2-a]pyridine derivative **1** to undergo annulations reactions with different reagents under different-reaction conditions. The biological assessment of compounds **2**, **11**, **14**, **15**, and **19** showed remarkable antimicrobial activities. In addition, selected derivatives of the products were screened for their anticancer activities against two tumor cell lines using MTT assay and the results showed that some of these compounds have potent cytotoxic effect, as concluded from their IC_{50} values. Meanwhile, compounds **3a**, **7** have exhibited very strong potency as anticancer candidates. Thiazolopyridine structures have been confirmed as a useful lead compounds for the development of new anticancer agents. Molecular docking showed that,-some of the synthesized compounds more suitable inhibitor against-ALR2 with farther alteration in future.

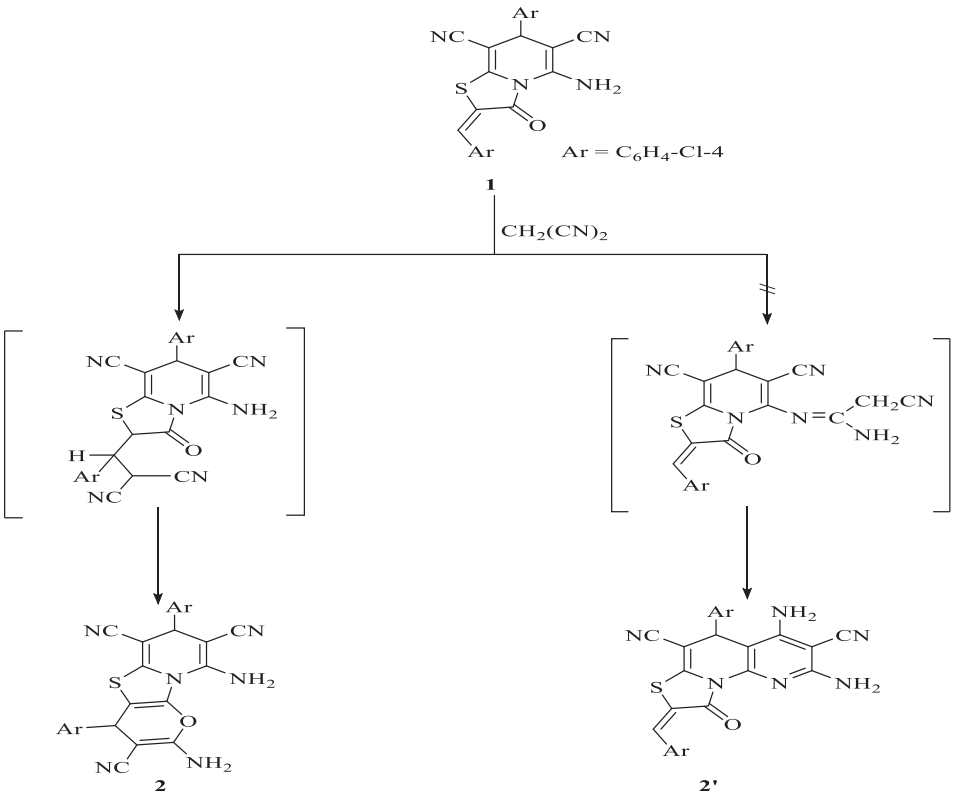
1 | INTRODUCTION

Heterocyclic systems have attracted a great attention in the last decade due to their importance in pharmaceutical and medicinal chemistry. One of these heterocycles is the thiazolidin-4-one scaffold which is considered as a backbone for the synthesis of different synthetic pharmaceuticals targets having a wide spectrum of biological activities.[1–8] On the other hand, literaturally nicotinonitrile moiety also plays a significant role in drug design field.[9–17] Therefore, the combination of new ring systems involving thiazolo[3,2-a]-pyridine derivatives showed remarkable antimicrobial,[18–23] antifungal,[24] anticancer,[25–32] and antihypertensive activities.[33]

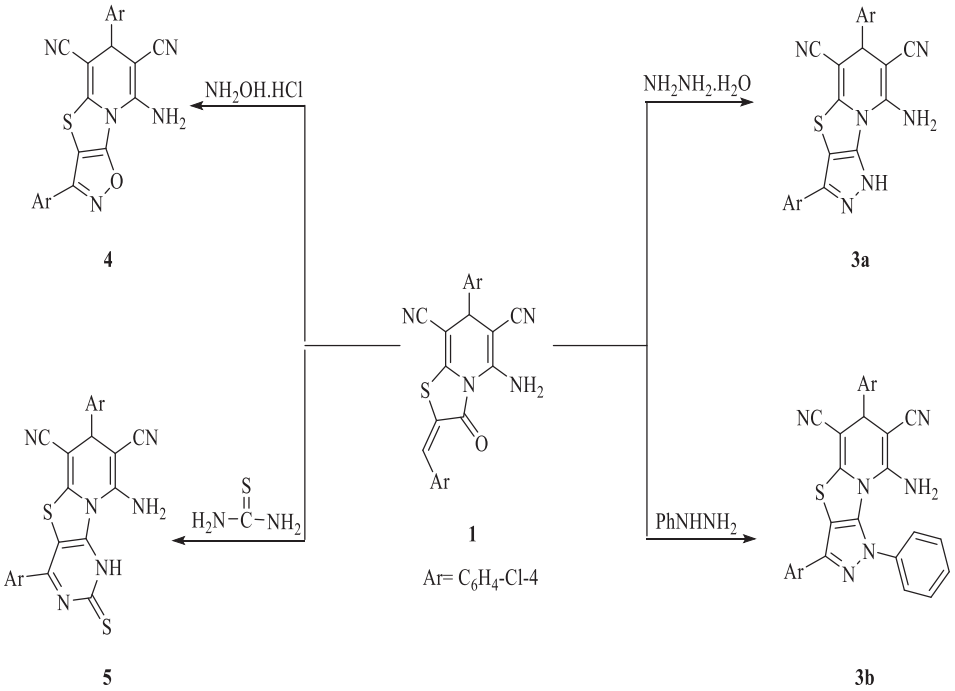
Due to all the aforementioned facts and in continuation of our program regarding the synthesis of biologically active heterocyclic nuclei.[34–37] Herein, we used 5-aminothiazolo[3,2-a]pyridine derivative **1**, as a key starting material to construct different novel heterocyclic compounds. The structures of the newly synthesized compounds were ascertained from the spectral and elemental analyses. Some of the new compounds showed remarkable biological activities.

2 | RESULTS AND DISCUSSION

The strategy for the construction of different heterocycles bearing fused thiazolo[3,2-a]pyridine was emanated from the utilization of 5-amino-2-(4-chlorobenzylidene)-7-(4-chlorophenyl)-3-oxo-2,3-dihydro-7H-thiazolo[3,2-a]pyridine-6,8-dicarbonitrile **1** as the building block.[38] In the present work, the behavior of **1** towards malononitrile was investigated. Therefore, compound **1** was allowed to react with malononitrile in refluxing pyridine as shown in (Scheme 1). Supposedly, two possible products **2** and **2′** were anticipated to be yielded. When the reaction was monitored with thin-layer chromatography (TLC), it revealed the presence of one spot. Unambiguously, both the elemental analysis and spectral-data confirmed structure



SCHEME 1 Reaction of **1** with malononitrile

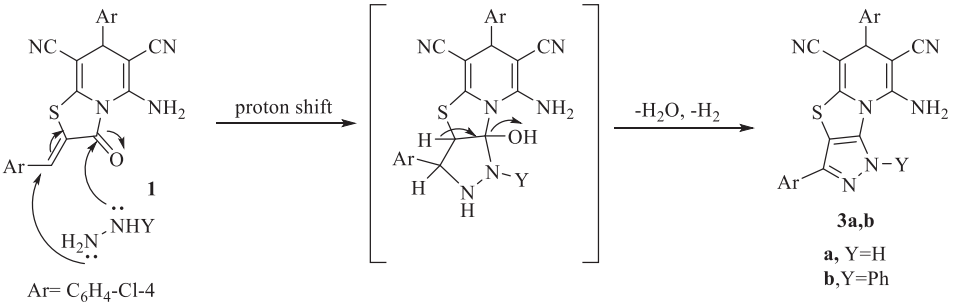


SCHEME 2 Reactions of **1** with different binucleophilic reagents

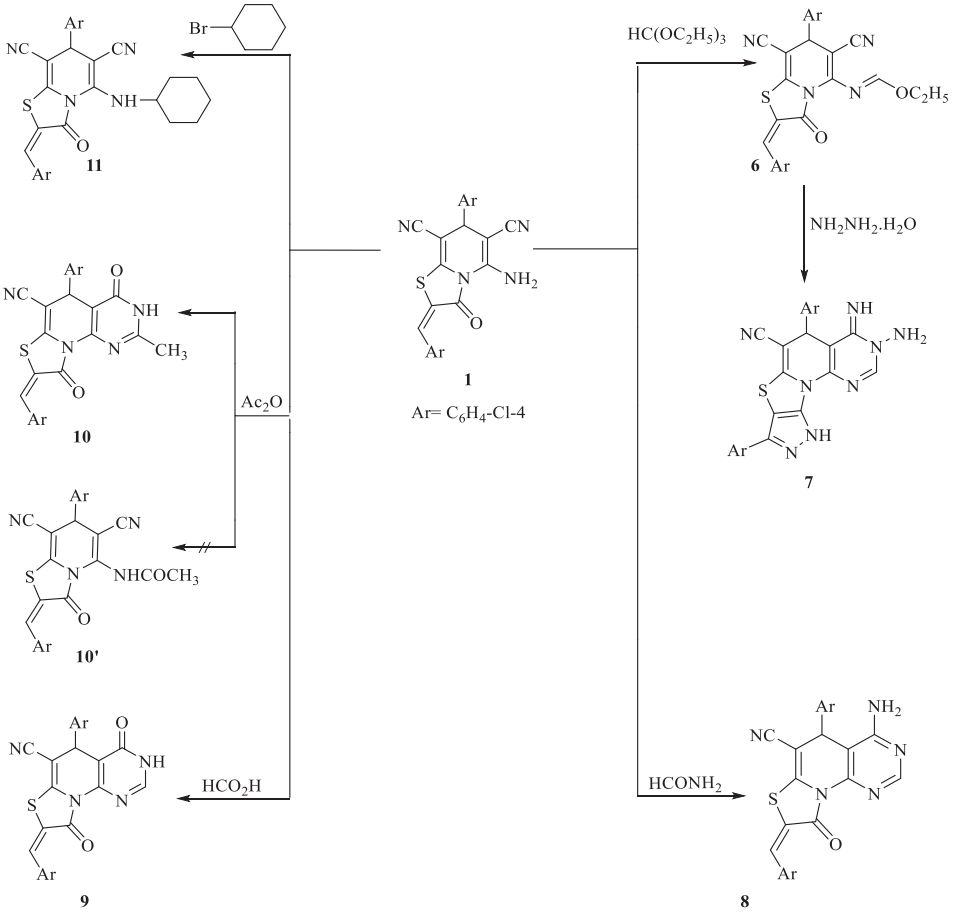
2 and excluded structure **2′**. Compound **2** was obtained by Michael addition on the α,β-unsaturated enone of **1** followed by intramolecular nucleophilic reaction of the enol form on the cyano group as shown in Scheme 1. As evidences, IR-spectrum revealed the absence of any absorption band corresponding to C═O group of the thiazolinone ring while ^{1}H-NMR showed singlet signal at $\delta = 3.61$ ppm due pyrane proton.

Treatment of an ethanolic solution of **1** with different binucleophilic agents such as hydrazine hydrate and/or phenyl hydrazine under reflux gave the corresponding pyrazolo[3,4:4,5]thiazolo[3,2-a]pyridine derivatives **3a** and

SCHEME 3 Mechanistic illustration for **3a** and **3b** formations

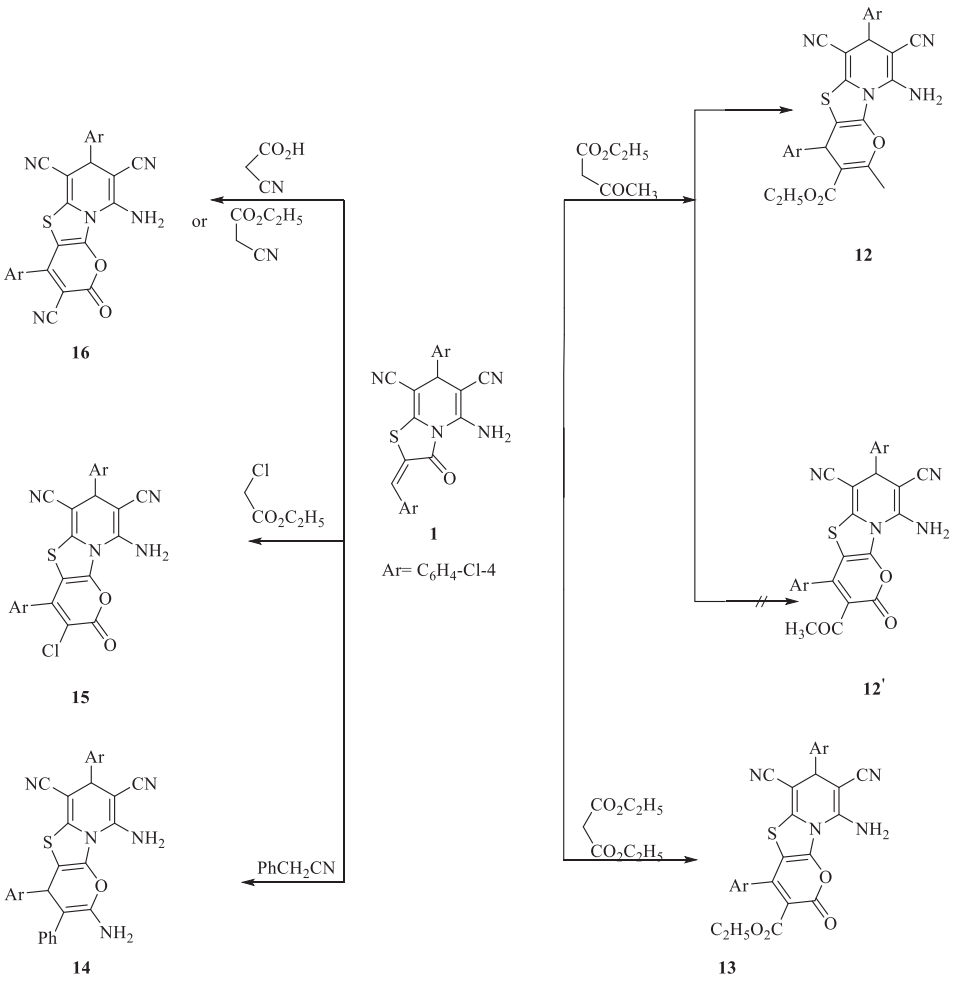


SCHEME 4 Reactions of **1** with different electrophilic agents



3b, respectively (Scheme 2). The IR-spectra of **3a** and **3b** lack any carbonyl signals and ^{1}H-NMR of compound **3a** showed a broad singlet signal at $\delta = 10.81$ ppm corresponding to the NH group of the pyrazole moiety. On the other hand, the mass spectrum of compound **3b** showed a peak at $m/z = 539$ ($M^{+\cdot} + 1$). A mechanistic illustration for **3a** and **3b** formation commenced with intramolecular-cyclocondensation with α,β-unsaturated carbonyl system of **1** followed by oxidation to give **3a** and **3b** as depicted in Scheme 3. As alternative binucleophilic agents, both of hydroxylamine hydrochloride and thiourea were subjected to react with **1** to yield isoxazolo[5′,4′:4,5] thiazolo[3,2-a]pyridine derivatives **4** and **5,** respectively. IR-spectra of compounds **4** and **5** lacked any carbonyl group absorption band. ^{1}H-NMR of compound **5** showed a broad singlet signal at $\delta = 10.38$ ppm due to NH pyrimidine.

In order to construct the four-ring fused system pyrazolo[3′,4′:4′,5′]thiazolo [3′,2′:1,6]pyrido[2,3-d]pyrimidine-6-carbonitrile **7**, the thiazolopyridine derivative **1** was subjected to react with triethyl orthoformate under reflux condition to give the corresponding derivative **6**, which in turn was hydrazonolyzed with hydrazine hydrate to get the target compound **7** (Scheme 4). The ^{1}H-NMR spectrum compound **6** lacked any characteristic band for the amino group and appearance of ethoxy signals at $\delta = 4.37$ and 1.36 ppm. The ^{1}H-NMR spectrum of



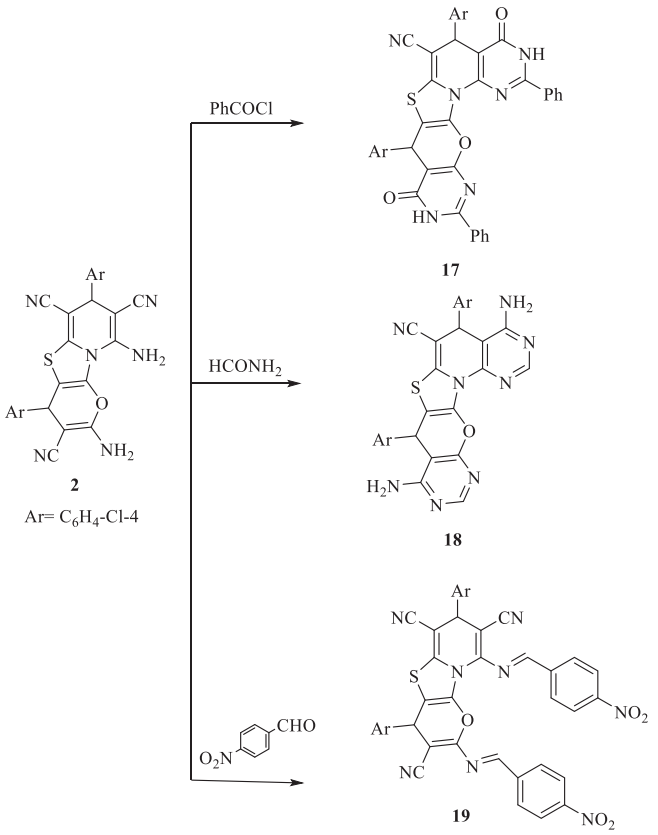
SCHEME 5 Reactions of **1** with different active methylene compounds

compound **7** showed an exchangeable broad singlet band of NH_2 protons at $\delta = 5.18$ ppm besides two exchangeable-broad singlet signals at $\delta = 12.79$ and 11.15 ppm due to 2NH protons. Furthermore, **1** gave the corresponding thiazolopyrido pyrimidine derivatives **8** and **9** by treatment with formamide and formic acid, respectively (Scheme 4). The mass spectra of compounds **8** and **9** revealed the molecular ion peaks.

On the other hand, when **1** was refluxed with acetic anhydride, the thiazolopyrido pyrimidine derivative **10** was yielded. The structure of **10** was confirmed by the spectral data such as the IR-spectrum which exhibited strong absorption bands at $\upsilon = 3428\ cm^{-1}$ (NH), $2214\ cm^{-1}$ (C≡N) and 1709, $1675\ cm^{-1}$ (2C═O). The ^{1}H-NMR spectrum exhibited a singlet signal at $\delta = 1.91$ ppm assigned to the methyl protons.

Nucleophilic substitution of **1** with cyclohexyl bromide yielded cyclohexylamino-thiazolo[3,2-a]pyridine derivative **11** (Scheme 4). The structure of compound **11** was confirmed by the IR-spectrum which exhibited an absorption band at $\upsilon = 3204\ cm^{-1}$ (NH). ^{1}H-NMR of compound **11** showed D_2O-exchangeable singlet signal at $\delta = 8.98$ ppm due to NH proton and multiplet signals due to cyclohexyl protons at 2.16 to 1.62 ppm.

Pyrano[2′,3′:4,5]thiazolo[3,2-a]pyridine derivatives **12**, **13**, **14**, **15**, and **16** were obtained when compound **1** was reacted with different active methylene compounds such as ethyl acetoacetate, diethyl malonate, benzyl cyanide, ethyl chloroacetate, cyanoacetic acid and/or ethyl cyanoacetate, respectively (Scheme 5). Structural elucidations of formed compounds were confirmed by exploiting their spectral data. For instance, the IR-spectrum of compound **12** exhibited a strong absorption band at $\upsilon = 1725\ cm^{-1}$ corresponding to (C═O) group while its ^{1}H-NMR showed bands at $\delta = 4.10$ and 1.25 ppm corresponding to ethyl group protons. Finally, the mass spectrum revealed the molecular ion peak at m/z 562. For compound **13**, the IR-spectrum of the compound showed absorption bands at 1771 and $1710\ cm^{-1}$ due to two C═O groups. In addition, its ^{1}H-NMR showed bands at $\delta = 4.21$ and 1.2 ppm corresponding to ethyl group protons. The IR spectrum of compound **14** showed the absence of any absorption band of C═O (thiazolidinone). ^{1}H-NMR of compound **14** showed-D_2O-exchangeable



SCHEME 6 Reactions of 2 with benzoyl chloride, formamide, and *p*-nitrobenzaldehyde

singlet signals at $\delta = 5.09$ and 4.65 ppm due to four protons of $2NH_2$ groups. The mass spectra of compounds **15** and **16** revealed the molecular ion peaks at m/z 524 and m/z 512, respectively.

For exploring the utilization of the pyrano[2′,3′:4,5] thiazolo[3,2-a]pyridine derivative **2** as an useful key intermediate in the synthesis of fused heterocyclic derivatives, initially it was treated with benzoyl chloride and formamide to give the pyrimidino[3‴,4‴:5″,6″]pyrano[2″,3″:4′,5′] thiazolo[3′,2′:1,6]pyrido[2,3-d]pyrimidine derivatives **17** and **18**, respectively (Scheme 6). The structure of **17** was confirmed by the spectral data such as the IR which lacked any band corresponding to the amino group. As an additional proof, ^{1}H-NMR spectrum showed the absence of any broad singlet signals of the two NH_2 protons and showed D_2O-exchangeable singlet signal at $\delta = 12.9$ ppm corresponding to the two NH protons. The mass spectrum of compound **18** showed its molecular ion peak at m/z 567.

Eventually, the reactivities of both amino groups in **2** has been proved to be comparable. This comparison was carried out by allowing **2** to react with the electrophilic agent 4-nitrobenzaldehyde. When **2** was treated with one equivalent and/or two equivalents of 4-nitrobenzaldehyde, in both cases the bis Schiff base **19** was yielded with different yields (25% and 60% respectively). The mass spectrum of **19** showed a peak at m/z 751 ($M^{+\cdot} - 32$).

2.1 | Antibacterial activity evaluation

The antimicrobial activities of the new compounds have been screened using-agar diffusion method,[39,40] against *Staphylococcus aureus* (G+ve), *Pseudomonas aeruginosa* (G−ve), *Candida-albicans* (yeast), and *Aspergillus niger* (fungus). As compiled in Table 1, it is obvious that most of the tested compounds showed moderate to high antimicrobial activities. In particular, compounds **2, 11**, **14, 15**, and **19** showed remarkable antimicrobial activities. It is promising to consider those derivatives are having the chance to be candidates as antimicrobial agents.

2.2 | Molecular docking studies

Molecular docking study of thiazolopyridine derivatives was performed by Molecular Operating Environment 2014.09.[41–43] In order to understand the binding mode of protein-ligand interactions, the crystal structure of the human-ALR2 enzyme (1PWM) complexed with (ligand Fidarestat) as an inhibitor was downloaded from a protein data bank. The molecular docking scoring function was applied to-evaluate the binding affinities between the (1PWM) and selected synthesized inhibitors (FID) was re-docked into the active site of the enzyme, and then replaced it with the tested derivatives in order to compare the binding mode of ligand and the tested derivatives (Figures 1-10, Table 2). We begin to carry out a molecular docking study of the highest biologically active newly synthesized derivatives. First, the enzyme prepared for docking by adding lost hydrogen, reconnect the bonds broken and fixing the-potential, also active sites has been identified and we start to replace the known ligand (FID) by our synthesized ligands.

The results of docking study were reported as -ALR2 (1PWM) binding free energy (δ*G*). The negative values of free energies refer to spontaneity of bindings (Table 2). The docking results revealed that the highest binding compound to ALR2 enzyme was **18** with binding energy of −7.61 kcal/mol, this was attributed to the strong hydrogen bond between the ligand and Lys 21, Ser 263, and Asn 272 from the enzyme and arenes interaction of Leu228 and Arg 268. On the other hand, compound **14** also exhibit promising ALR2 enzyme inhibitory activity (−7.16 kcal/mol). It showed hydrogen bond between the ligand and Lys 21 and Asn 272. Finally, compound **2** showed hydrogen bond between the ligand and Lys

TABLE 1 Antimicrobial evaluations of the synthesized compounds

Sample number	Clear zone (ϕ mm)			
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Candida albicans*	*Aspergillus niger*
1	16	18	17	13
2	28	30	16	15
3a	11	18	18	0
3b	14	11	14	0
4	12	15	18	0
5	14	16	15	0
7	15	18	14	0
8	17	14	17	0
10	14	21	15	14
11	25	22	27	0
12	13	10	15	13
14	21	22	18	16
15	29	31	31	13
16	12	13	0	0
17	17	14	0	0
18	15	18	0	15
19	22	23	23	10
Streptomycin	30	28	25	15
Cyclohexamide	00	00	22	37

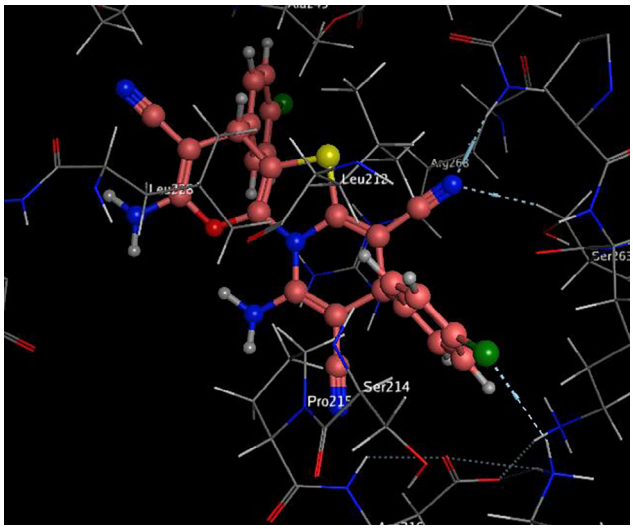


FIGURE 1 3D diagram of the interaction of compound **2** in the active site of the ALR2 enzyme

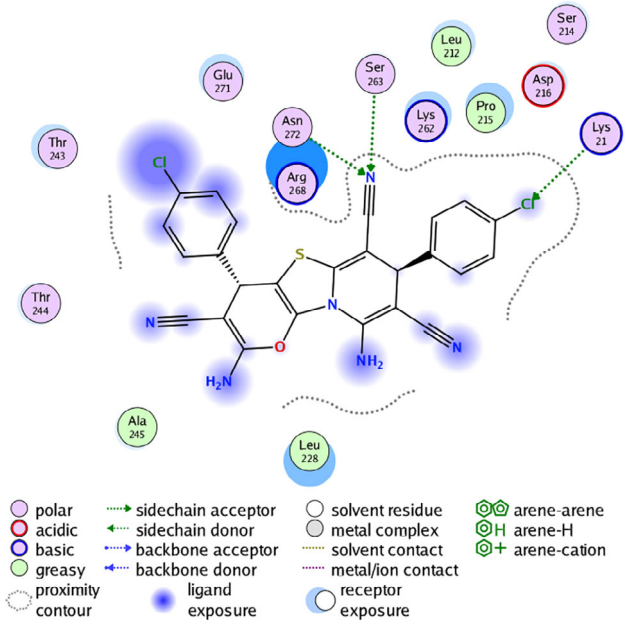


FIGURE 2 2D diagram of the interaction of compound **2** in the active site of the ALR2 enzyme

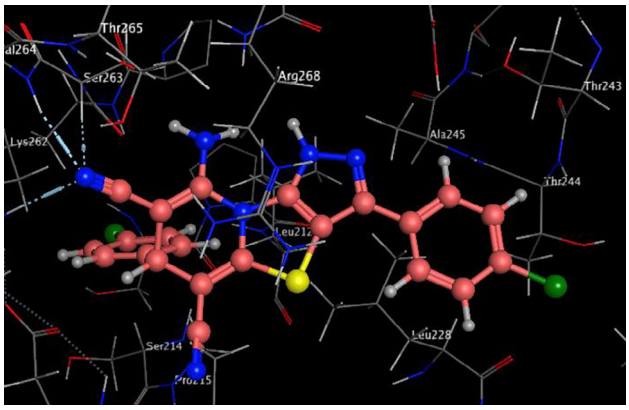


FIGURE 3 3D diagram of the interaction of compound **3a** in the active site of the ALR2 enzyme

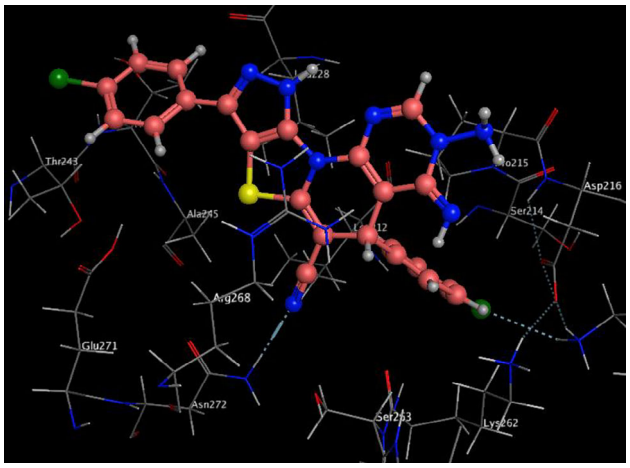


FIGURE 5 3D diagram of the interaction of compound **7** in the active site of the ALR2 enzyme

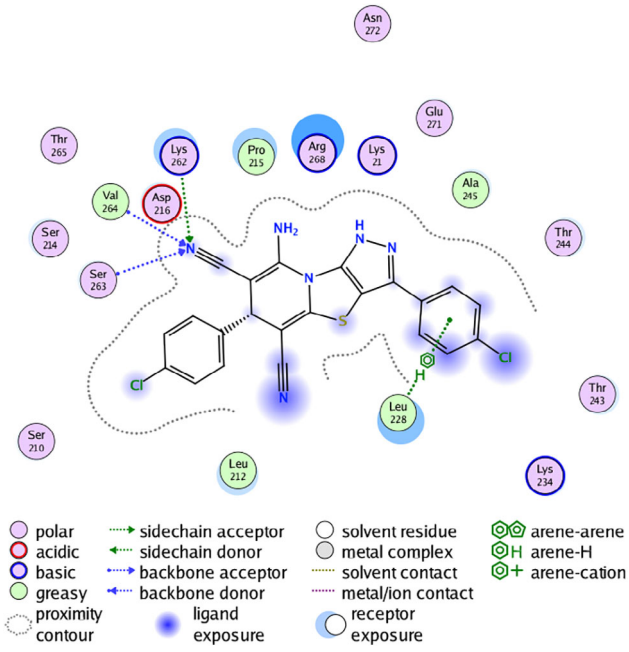


FIGURE 4 2D diagram of the interaction of compound **3a** in the active site of the ALR2 enzyme

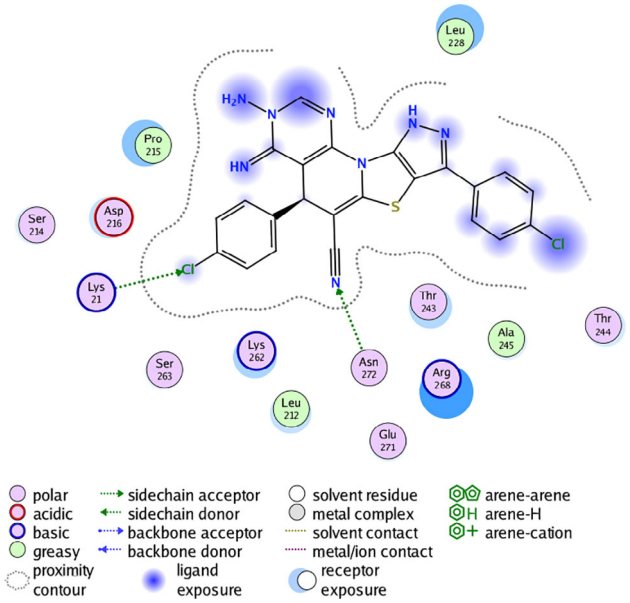


FIGURE 6 2D diagram of the interaction of compound **7** in the active site of the ALR2 enzyme

21, Ser 263 and Asn 272 that makes ALR2 enzyme inhibitory activity (−7.11 kcal/mol).

2.3 | Antiproliferative activities

The results of the cytotoxic activity in vitro were expressed as the IC_{50}-concentration of the compound (in μM) that inhibits proliferation of the cells by 50% as compared to the untreated control cells. The synthesized compounds are tested for anticancer activities against two human tumor cells,[44,45] namely hepatocellular carcinoma (HepG-2) and mammary gland (MCF-7). The results on the antiproliferative activity of tested compounds are summarized in (Table 3**)**, the synthesized compounds which exhibited very strong antiproliferative activity are **3a** and **7** with IC_{50}: 6.10, 7.80 and 9.43, 8.24 μg against two carcinoma cell lines tested in this study HepG-2 and MCF-7 cell lines, respectively, which are comparable with the reference anticancer drug Doxorubicin (DOX) with IC_{50}: 4.50 and 4.17 μg. Compounds **2, 14**, and **18** also showed strong anticancer activity against two carcinoma cell lines tested in this study with IC_{50}: (19.04, 10.21), (13.91, 12. 65) and (16.28, 14.06) μg for HepG-2 and MCF-7 cell lines, respectively. While the other

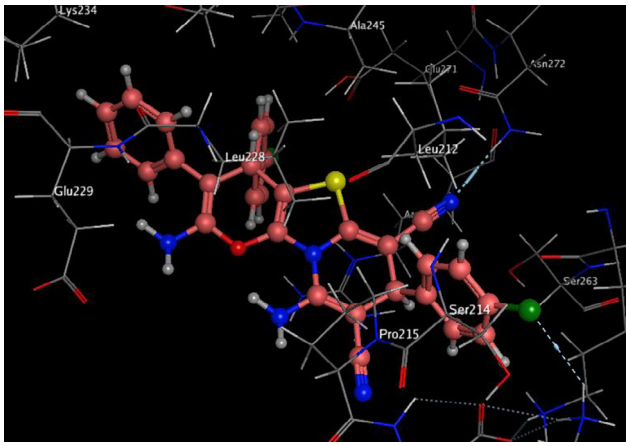


FIGURE 7 3D diagram of the interaction of compound **14** in the active site of the ALR2 enzyme

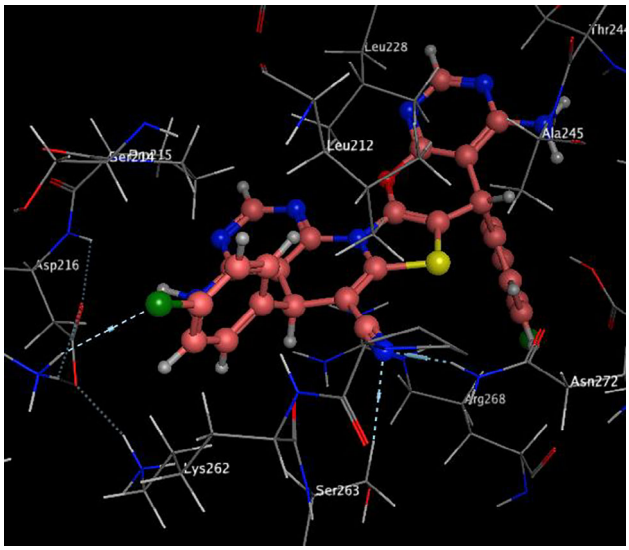


FIGURE 9 3D diagram of the interaction of compound **18** in the active site of the ALR2 enzyme

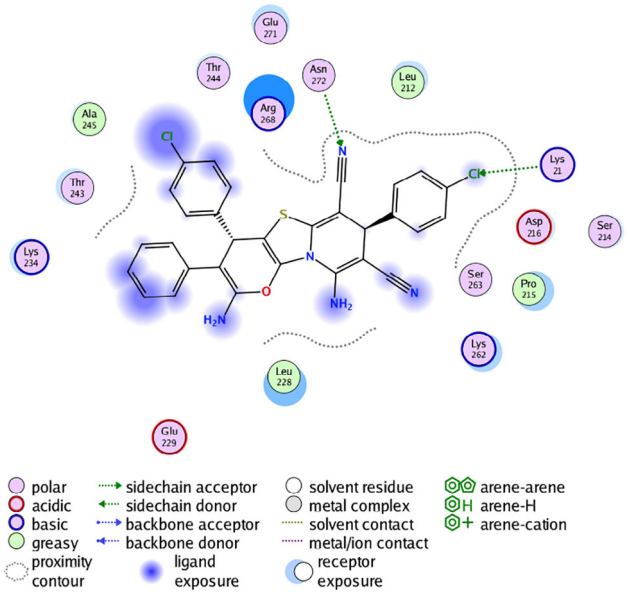


FIGURE 8 2D diagram of the interaction of compound **14** in the active site of the ALR2 enzyme

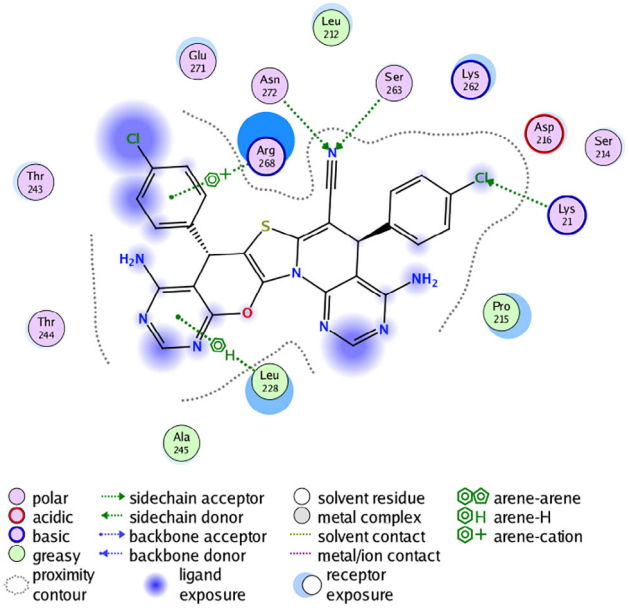


FIGURE 10 2D diagram of the interaction of compound **18** in the active site of the ALR2 enzyme

TABLE 2 The docking binding free energies of the synthesized compounds

Compound number	Docking score (kcal/mol)	No. of hydrogen bonding	No. of (arenes) interaction
FID	−6.24	2 (Lys 262 and Asn 272)	—
2	−7.11	3 (Lys 21, Ser 263 and Asn 272)	—
3a	−6.39	3 (Lys 262, Ser 263 and Val 264)	1 (Leu 228) (arene-H)
7	−6.91	2 (Lys 21 and Asn 272)	—
14	−7.16	2 (Lys 21 and Asn 272)	—
18	−7.61	3 (Lys 21, Ser 263 and Asn 272)	1 (Leu 228) (arene-H); 1 (Arg 268) (arene-+)

compounds that showed moderate antiproliferative activity are **3b**, **4**, **5**, **8**, **11**, **13**, and**16**; compounds **1**, **10**, **12**, **15**, and **19** show weak cytotoxicity activity against all cell lines (Table 3). In conclusion, thiazolopyridine structures have been confirmed as a useful lead compounds for the development of new anticancer agents. Our initial goal to prepare synthetic derivatives with higher anticancer activity could be achieved.

Graphical correlation between tested compounds and their IC_{50} against HePG-2 and MCF-7 is depicted in Figure 11.

TABLE 3 Antiproliferative activities of some compounds against human tumor cells

	In vitro cytotoxicity IC_{50} (μM)[a]	
Compound number	**HePG-2**	**MCF-7**
Doxorubicin	4.50 ± 0.3	4.17 ± 0.2
1	87.18 ± 4.5	85.32 ± 4.3
2	19.04 ± 1.7	10.21 ± 1.0
3a	6.10 ± 0.5	7.80 ± 0.7
3b	48.17 ± 2.9	42.17 ± 2.6
4	35.49 ± 2.4	26.67 ± 2.1
5	23.84 ± 1.9	17.23 ± 1.7
7	9.43 ± 0.8	8.24 ± 0.8
8	35.81 ± 2.3	29.01 ± 2.2
10	82.76 ± 4.0	65.49 ± 3.4
11	28.22 ± 2.1	21.15 ± 1.9
12	54.39 ± 3.2	49.79 ± 2.8
14	13.91 ± 1.1	12.65 ± 1.2
15	64.54 ± 3.4	53.58 ± 3.1
16	42.72 ± 2.7	33.88 ± 2.4
17	>100	91.14 ± 5.1
18	16.28 ± 1.3	14.06 ± 1.4
19	72.35 ± 3.6	78.26 ± 3.8

[a]IC_{50} (μM): 1-10 (very-strong), 11-20 (strong), 21-50 (moderate), 51-100 (weak), and above 100 (non-cytotoxic).

2.4 | Structure activity relationship

Upon investigations of the results revealed from the assessment of the tested compounds as inhibitors against two different tumor cell lines, it is obvious that activities are dependent on existence of certain structural features of the tested compounds. The most cytotoxic derivatives were found in **2**, **3a**, **14**, and **18** since it has potent which may attribute to the presence of the amino and imino groups. Therefore, as a conclusion, derivatives with more than one amino and imino group exhibited remarkable activities as anti-tumor candidates when compared with those having one only (Figures 12 and 13).

3 | CONCLUSIONS

The present work aimed to the development of novel molecules containing thiazolopyridine moieties. The biological assessment of compounds **2**, **11**, **14**, **15**, and **19** showed remarkable antimicrobial activities. Meanwhile, selected derivatives of the products were screened for their anticancer activities against two tumor cell lines using MTT assay and the results showed that some of these compounds **3a**, **7** have exhibited very strong potency as anticancer candidates. Thiazolopyridine structures have been confirmed

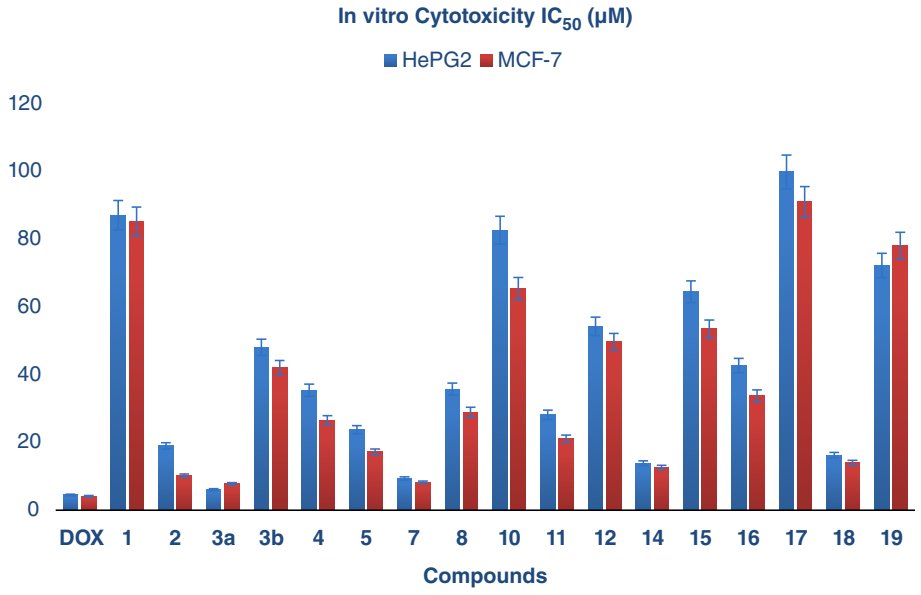


FIGURE 11 Graphical correlation between tested compounds and their IC_{50} against HePG-2 and MCF-7

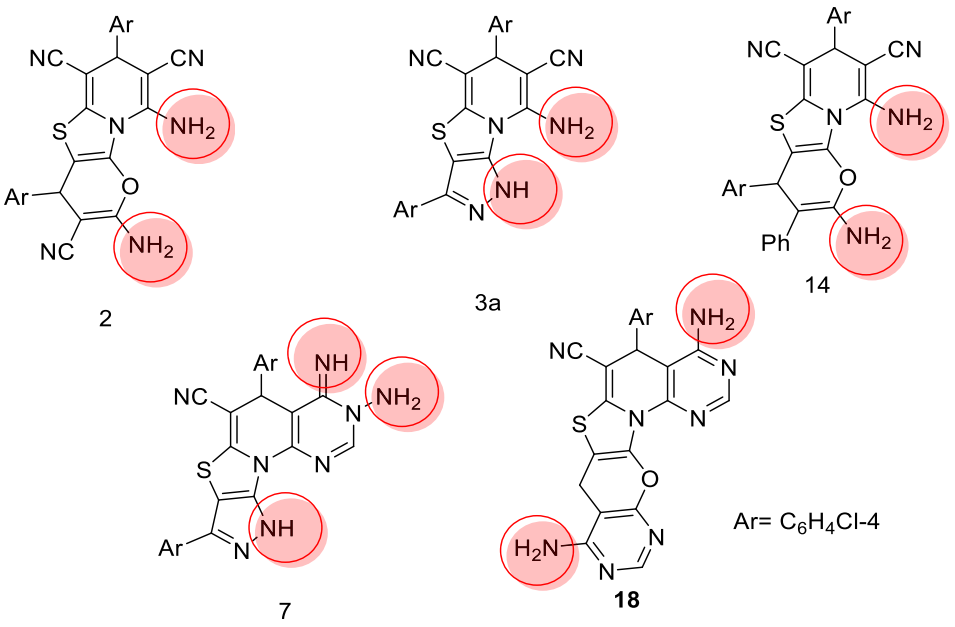


FIGURE 12 High anticancer activities

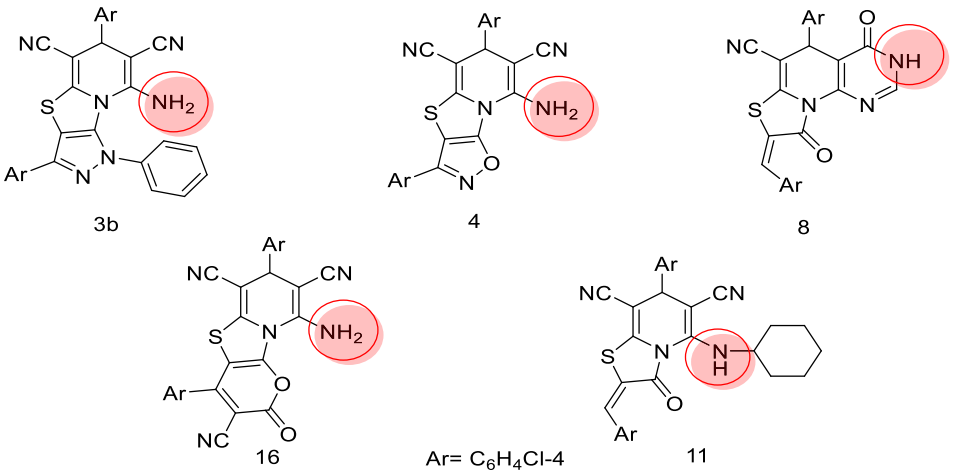


FIGURE 13 Moderate anticancer activities

as a useful lead compounds for the development of new anticancer agents. Molecular docking showed that some of the synthesized compounds are more suitable inhibitor against ALR2 with farther alteration in future.

4 | EXPERIMENTAL

Melting points were measured on a Griffin melting point apparatus. The IR spectra were measured with a Pye Unicam SP 2000 infrared spectrophotometer using the KBr wafer technique. The EI-MS spectra were determined using an AE1 MS 902 mass spectrometer. The ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) were measured on Varian Gemini instrument with chemical shifts (δ) expressed in ppm downfield from TMS as internal standard in deuterated dimethylsulfoxide (DMSO-d_6). All the spectral measurements were performed at the Micro Analytical Center of Cairo University, Egypt, the Micro Analytical-Center of Ain-Shams University, or the Main Defense Chemical Laboratory. Elemental analyses were realized at the Faculty of Science, Ain Shams University. The antimicrobial activities were determined at Al-Azhar University, Faculty of Science. The anticancer evaluation was performed at the Pharmacology Department, Faculty of Pharmacy, Mansoura University, Egypt. The chemical reactions were monitored by TLC.

4.1 | 5-Amino-2-(4-chlorobenzylidene)-7-(4-chlorophenyl)-3-oxo-2,3-dihydro-7H-thiazolo[3,2-a]pyridine-6,8-dicarbonitrile (1)

A mixture of 4-chlorobenzaldehyde (0.02 mol, 2.81 g), malononitrile (0.02 mol, 1.32 g) and thioglycolic acid

(0.01 mol, 0.92 mL) in absolute ethanol containing drops of triethyl amine (5 drops) was heated under reflux for 2 hours. The formed precipitate was filtered off, washed with ethanol and recrystallized from dioxane to give **1**.

Yield (75%), mp 268°C-270°C, (dioxane). Anal. Calcd. For $C_{22}H_{12}Cl_2N_4OS$ (451): C, 58.55; H, 2.68; N, 12.41. Found: C, 58.52; H, 2.64; N, 12.46. FT-IR (KBr) (cm^{-1}): 3379, 3284 (NH_2), 2208 (CN), 1719 (C═O). ^{1}H-NMR (DMSO-d_6): δ 7.84 (br.s, 2*H*, NH_2, D_2O exchangeable), 7.66 (s, 1*H*, CH-arylidine), 7.54-7.47 (m, 8*H*, H-Ar), and 4.65 (s, 1*H*, H pyridine). MS, *m*/*z* (%): 451 ($M^{\cdot}$+, 8.3%).

4.2 | 2,9-Diamino-4,7-bis (4-chlorophenyl)-4H,7H-pyrano[2′,3′:4,5] thiazolo[3,2-a]pyridine-3,6,8-tricarbonitrile (2)

A mixture of compound **1** (0.01 mol, 4.51 g) and malononitrile (0.01 mol, 0.66 g) in dry pyridine (20 mL) was heated under reflux for 10 hours. The reaction mixture was poured into ice/HCl. The formed precipitate was filtered off, dried and recrystallized from ethanol to give **2**.

Yield (44%), mp >300°C (ethanol). Anal. Calcd. For $C_{25}H_{14}C_{l2}N_6OS$ (517): C, 58.04; H, 2.73; N, 16.24. Found: C, 58.06; H, 2.70; N, 16.26. FT-IR (KBr) (cm^{-1}): 3406-3156 ($2NH_2$), 2211 (CN). ^{1}H-NMR (DMSO-d_6) δ: 7.90-7.31 (m, 8*H*, H-Ar), 6.61 (br.s, 2*H*, NH_2, exchangeable), 6.49 (br.s, 2*H*, NH_2, D_2O exchangeable), 4.10 (s, 1*H*, H-pyridine), 3.61 (s, 1*H*, H-pyrane). MS, *m*/*z* (%): 517 ($M^{+\cdot}$, 37.6%).

4.3 | 8-Amino-3,6-bis(4-chlorophenyl)-1H,6H-pyrazolo[3′,4′:4,5]thiazolo[3,2-a] pyridine-5,7-dicarbonitrile (3a)

A mixture of compound **1** (0.01 mol, 4.51 g), hydrazine hydrate (0.02 mol, 1 mL) in absolute ethanol (30 mL) was heated under reflux for 20 hours. The reaction mixture was poured into ice/HCl. The formed precipitate was filtered off, dried and recrystallized from mixed benzene/ethanol to give **3a**.

Yield (70%), mp 112°C-114°C (benzene/ethanol). Anal. Calcd. For $C_{22}H_{12}Cl_2N_6S$ (463): C, 57.03; H, 2.61; N, 18.14; Found: C, 57.01; H, 2.63; N, 18.13. FT-IR (KBr) (cm^{-1}): 3329-3216 (NH_2, NH), 2211 (CN), 1599 (C═N). ^{1}H-NMR (DMSO-d_6) δ: 10.81 (br.s, 1*H*, NH, D_2O exchangeable), 8.04-7.17 (m, 8*H*, H-Ar), 4.81 (br.s, 2*H*, NH_2, D_2O exchangeable), and 4.73 (s, 1*H*, H-pyridine). MS, *m*/*z* (%): 422 ($M^{+\cdot}$ − 41, 4.5%).

4.4 | 8-Amino-3,6-bis(4-chlorophenyl)-1-phenyl-1H,6H-pyrazolo[3′,4′:4,5]thiazolo [3,2-a]pyridine-5,7-dicarbonitrile (3b)

To a solution of compound **1** (0.01 mol, 4.51 g) in absolute ethanol (20 mL) phenyl hydrazine (0.02 mol, 2 mL) was added in the presence of drops of piperidine (5 drops). The reaction mixture was heated under reflux for 20 hours. The reaction mixture was poured into ice/HCl. The formed precipitate was filtered off, dried and recrystallized from dioxane to give **3b**.

Yield (64%), mp >300°C (dioxane). Anal. Calcd. For $C_{28}H_{16}Cl_2N_6S$ (538): C, 62.34; H, 2.99; N, 15.58. Found: C, 62.31; H, 3.03; N, 15.61. FT-IR (KBr) (cm^{-1}): 3476-3388 (NH_2), 2216 (CN), 1580 (C═N). ^{1}H-NMR (DMSO-d_6) δ: 7.82-7.27 (m, 13*H*, H-Ar), 6.28 (br.s, 2*H*, NH_2, D_2O exchangeable) and 4.65 (s, 1*H*, H-pyridine). ^{13}C-NMR (DMSO-d_6) δ: 161.5, 156.4, 151.7, 142.1, 140.0, 139.4, 134.6, 131.8, 131.6, 130.3 (2), 129.3 (4), 128.9 (2), 128.5 (2), 126.0, 123.3 (2), 118.3, 117.4, 93.0, 61.4, 57.6, 37.1. MS, *m*/*z* (%): 539 ($M^{+\cdot}$ + 1, 1.5%).

4.5 | Amino-3,6-bis(4-chlorophenyl)-6H-isoxazolo[5′,4′:4,5]thiazolo[3,2-a]pyridine-5,7-dicarbonitrile (4)

A mixture of compound **1** (0.01 mol, 4.51 g) and hydroxylamine hydrochloride (0.01 mol, 0.68 g) in alcoholic NaOH (20%) (30 mL) was heated under reflux for 20 hours. The reaction mixture was poured into ice/HCl. The formed residue was filtered off, dried and recrystallized from benzene/ethanol to give **4**.

Yield (54%), mp 96°C-98°C (benzene/ethanol). Anal. Calcd. For $C_{22}H_{11}Cl_2N_5OS$ (464): C, 56.91; H, 2.39; N, 15.08. Found: C, 56.89; H, 2.37; N, 15.11. FT-IR (KBr) (cm^{-1}): broad band centered at 3442 (NH_2), 2207 (CN), 1645 (C═C), 1594 (C═C). ^{1}H-NMR (DMSO-d_6) δ: 7.82-7.50 (m, 8*H*, H-Ar), 5.57 (br.s, 2*H*, NH_2, D_2O exchangeable), and 4.15 (s, 1*H*, H- pyridine). MS, *m*/*z* (%): 464 ($M^{+\cdot}$, 16%).

4.6 | Amino-4,7-bis(4-chlorophenyl)-2-thioxo-1,2-dihydro-7H-pyrido[2′,1′:2,3] thiazolo [4,5-d]pyrimidine-6,8-dicarbonitrile (5)

A solution of compound **1** (0.01 mol, 4.51 g), thiourea (0.01 mol, 0.76 g) in ethyl alcohol (20 mL) and alc. sodium hydroxide (40%) (30 mL) was heated under reflux for 10 hours. The reaction mixture was poured into ice/HCl. The formed solid was filtered off, washed by

water several times, dried and recrystallized from benzene to give **5**.

Yield (66%), mp 184°C-186°C (benzene). Anal. Calcd. For $C_{23}H_{12}Cl_2N_6S_2$ (507): C, 54.44; H, 2.38; N, 16.56 Found: C, 54.43; H, 2.36; N, 16.62. FT-IR (KBr) (cm^{-1}): 3342-3192 (NH_2, NH), 2193 (CN), 1651 (C═N). ^{1}H-NMR (DMSO-d_6) δ: 10.38 (br.s, 1*H*, NH, D_2O exchangeable), 7.95 (br.s, 2*H*, NH_2, D_2O exchangeable), 7.68-7.08 (m, 8*H*, H-Ar), and 4.41 (s, 1*H*, H-pyridine). ^{13}C-NMR (DMSO-d_6) δ, 180.4, 164.6, 161.5, 151.7, 140.3, 138.4, 136.6, 131.8, 131.2, 130.6 (2), 130.4, 128.9 (2), 128.5 (2), 126.0, 118.3, 117.1, 80.0, 69.9, 57.6., 37.1. MS, *m*/*z* (%): 444 ($M^{+\cdot}$ − 62, 7%).

4.7 | Ethyl-*N*-(2-(-4-chlorobenzylidene)-7-(4-chlorophenyl)-6,8-dicyano-3-oxo-2,3-dihydro-7H-thiazolo[3,2-a]pyridin-5-yl) formimidate (6)

A solution of compound **1** (0.01 mol, 4.51 g) in triethyl orthoformate (20 mL) was heated under reflux for 6 hours. The reaction mixture was concentrated and then the formed precipitate was collected and recrystallized from ethanol to give **6**.

Yield (44%), mp 253°C-255°C (ethanol). Anal. Calcd. For $C_{25}H_{16}Cl_2N_4O_2S$ (507): C, 59.18; H, 3.18; N, 11.04. Found: C, 58.99; H, 3.12; N, 11.07. FT-IR (KBr) (cm^{-1}): 2209 (CN), 1739 (C═O), 1640 (C═N). ^{1}H-NMR (DMSO-d_6) δ: 8.22 (s, 1*H*, N═CH), 7.82-7.53 (m, 8*H*, H-Ar), 6.79 (s, 1*H*, CH-arylidene), 4.82 (s, 1*H*, H-pyridine), 4.37 (q, 2*H*, CH_2) 1.36 (t, 3*H*, CH_3). MS, *m*/*z* (%): 506 ($M^{+\cdot}$ − 1, 25%).

4.8 | 3-Amino-5,8-bis(4-chlorophenyl)-4-imino-3,5-dihydro-4H,10H-pyrazolo [3′,4′:4′,5′]thiazolo[3′,2′:1,6]pyrido[2,3-d] pyrimidine-6-carbonitrile (7)

Compound **6** (0.01 mol, 5.07 g) was fused with hydrazine hydrate (0.02 mol, 1 mL) for 8 hours. The reaction mixture was poured into ice/HCl and the formed precipitate was filtered off, dried and recrystallized from dioxane to yield **7**.

Yield (21%), mp >300°C (dioxane). Anal. Calcd. For $C_{23}H_{14}Cl_2N_8S$ (505): C, 54.66; H, 2.79; N, 22.17. Found: C, 54.69; H, 2.80; N, 22.22. FT-IR (KBr) (cm^{-1}): 3331-3211 (NH_2, 2NH), 2210 (CN), 1602 (C═N). ^{1}H-NMR (DMSO-d_6) δ: 12.79 (br.s, 1*H*, NH, D_2O exchangeable), 11.15 (br.s, 1*H*, NH, D_2O exchangeable), 8.36 (s, 1*H*, H-pyrimidine), 7.65-7.26 (m, 8*H*, H-Ar), 5.18 (br.s, 2*H*, NH_2, D_2O exchangeable), 4.27 (s, 1*H*, H-pyridine). MS, *m*/*z* (%): 505 ($M^{+\cdot}$, 14%).

4.9 | 4-Amino-8-(4-chlorobenzylidene)-5-(4-chlorophenyl)-9-oxo-8,9-dihydro-5H-thiazolo[3′,2′:1,6]pyrido[2,3-d]pyrimidine-6-carbonitrile (8)

A mixture of compound **1** (0.01 mol, 4.51 g) and formamide (20 mL) was heated under reflux for 12 hours. The reaction mixture was poured into ice/water and then the formed residue was filtered off, dried and recrystallized from ethanol to give **8**.

Yield (40%), mp >300°C (ethanol). Anal. Calcd. For $C_{23}H_{13}Cl_2N_5OS$ (478): C, 57.75; H, 2.74; N, 14.64. Found: C, 57.71; H, 2.77; N, 14.61. FT-IR (KBr) (cm^{-1}): 3332, 3203 (NH_2), 2207 (CN), 1714 (C═O), 1628 (C═N). MS, *m*/*z* (%): 480 ($M^{+\cdot}$ + 2, 20%).

4.10 | 8-(4-Chlorobenzylidene)-5-(4-chlorophenyl)-4,9-dioxo-3,5,8,9-tetrahydro-4H-thiazolo[3′,2′:1,6] pyrido[2,3-d]pyrimidine-6-carbonitrile (9)

A solution of compound **1** (0.01 mol, 4.51 g) in formic acid (20 mL) was heated under reflux for 10 hours. The reaction mixture was cooled, and the precipitated solid was collected by filtration and recrystallized from benzene to give **9**.

Yield (20%), mp >300°C (benzene). Anal. Calcd. For $C_{23}H_{12}Cl_2N_4O_2S$ (479): C, 57.63; H, 2.52; N, 11.69. Found: C, 57.65; H, 2.56; N, 11.68. FT-IR (KBr) (cm^{-1}): 3187 (NH), 2205 (CN), 1706, 1658 (2C═O), 1604 (C═N). MS, *m*/*z* (%): 479 ($M^{+\cdot}$, 41%).

4.11 | 8-(4-Chlorobenzylidene)-5-(4-chlorophenyl)-2-methyl-4,9-dioxo-3,5,8,9-tetrahydro-4H-thiazolo[3′,2′:1,6] pyrido[2,3-d]pyrimidine-6-carbonitrile (10)

A solution of compound **1** (0.01 mol, 4.51 g) in acetic anhydride (15 mL) and pyridine (5 mL) was heated under reflux for 24 hours. The reaction mixture was cooled and poured into ice/water with stirring and refrigerated overnight. The separated solid was collected by filtration, washed by water, dried and recrystallized from toluene to give **10**.

Yield (65%), mp >300°C (toluene). Anal. Calcd. For $C_{24}H_{14}Cl_2N_4O_2S$ (493): C, 58.43; H, 2.86; N, 11.36. Found: C, 58.40; H, 2.89; N, 11.40. FT-IR (KBr) (cm^{-1}):

3428 (NH), 2214 (CN), 1709 (C═O), 1675 (C═O), 1635 (C═N). ^{1}H-NMR (DMSO-d_6) δ: 12.33 (br.s, 1*H*, NH, D_2O exchangeable), 7.95 (s, 1*H*, CH-arylidene), 7.93-6.98 (m, 8*H*, H-Ar), 4.63 (s, 1*H*, H-pyridine), and 1.91 (s, 3*H*, CH_3). MS, *m*/*z* (%): 493 ($M^{+\cdot}$, 25%).

4.12 | 2-(4-Chlorobenzylidene)-7-(4-chlorophenyl)-5-(cyclohexylamino)-3-oxo-2,3-dihydro-7H-thiazolo[3,2-a]pyridine-6,8-dicarbonitrile (11)

A mixture of compound **1** (0.01 mol, 4.51 g) and bromocyclohexane (0.01 mol, 1.63 mL) in alcoholic sodium hydroxide (40%) (30 mL) was heated under reflux for 8 hours. The reaction mixture was poured into ice/HCl. The formed precipitate was filtered off, dried and recrystallized by benzene to give **11**.

Yield (33%), mp 295°C-296°C (benzene). Anal. Calcd. For $C_{28}H_{22}Cl_2N_4OS$ (533): C, 63.04; H, 4.16; N, 10.50. Found: C, 63.02; H, 4.19; N, 10.53. FT-IR (KBr) (cm^{-1}): 3204 (NH), 2188 (CN), 1719 (C═O), 1641 (C═C). ^{1}H-NMR (DMSO-d_6) δ: 8.98 (br.s, 1*H*, NH, D_2O exchangeable), 8.13 (s, 1*H*, CH-arylidene), 7.95-6.92 (m, 8*H*, H-Ar), 4.48 (s, 1*H*, H-pyridine), 2.16-1.62 (m, 11*H*, H-cyclohexane). ^{13}C-NMR (DMSO-d_6) δ, 161.5, 157.3, 152.0, 140.3, 138.4, 133.6, 133.3, 133.2, 131.3, 130.4 (2), 129.0 (2), 128.9 (3), 127.5, 118.3, 117.1, 70.5, 58.2, 57.6, 37.8, 32.9 (2), 25.7, 25.1 (2). MS, *m*/*z* (%): 530 ($M^{+\cdot} - 2$, 18%).

4.13 | The general method for preparing 12, 13, 15, 16

A solution of compound **1** (0.01 mol, 4.51 g) in sodium ethoxide (0.5 g of sodium in 25 mL of ethanol) and ethyl acetoacetate and/or diethyl malonate and/or benzyl cyanide and/or ethyl chloroacetate and/or ethyl cyanoacetate and/or cyano acetic acid (0.01 mol) was heated under reflux for 8 hours. The reaction mixture was poured into ice/HCl. The formed precipitate was filtered off, dried and recrystallized from proper solvent to yield **12**, **13**, **15**, and **16**, respectively.

4.14 | Ethyl 9-amino-4,7-bis(4-chlorophenyl)-6,8-dicyano-2-methyl-4H,7H-pyrano[2′,3′:4,5]thiazolo[3,2-a]pyridine-3-carboxylate (12)

Yield (30%), mp 285°C-287°C (ethanol). Anal. Calcd. For $C_{28}H_{20}Cl_2N_4O_3S$ (563): C, 59.69; H, 3.58; N, 9.94. Found: C, 59.67; H, 3.61; N, 9.97. FT-IR (KBr) (cm^{-1}): 3383-3217 (NH_2), 2214 (CN), 1725 (C═O), 1625 (C═C). ^{1}H-NMR (DMSO-d_6) δ: 7.95-7.01 (m, 8*H*, H-Ar), 6.14 (br. s, 2*H*, NH_2, D_2O exchangeable), 5.45 (s, 1*H*, H-pyridine), 5.10 (s, 1*H*, H-pyrane), 4.20 (q, 2*H*, CH_2), 2.01 (s, 3*H*, CH_3), 1.20 (t, 3*H*, CH_3). MS, *m*/*z* (%): 563 ($M^{+\cdot}$, 42%).

4.15 | Ethyl 9-amino-4,7-bis(4-chlorophenyl)-6,8-dicyano-2-oxo-2H,7H-pyrano[2′,3′:4,5]thiazolo[3,2-a]pyridine-3-carboxylate (13)

Yield (38%), mp 183°C-184°C (ethanol). Anal. Calcd. For $C_{27}H_{16}Cl_2N_4O_4S$ (563): C, 57.56; H, 2.86; N, 9.94. Found: C, 57.58; H, 2.84; N, 9.91. FT-IR (KBr) (cm^{-1}): 3332-3204 (NH_2), 2206 (CN), 1771 (C═O), 1710 (C═O), 1609 (C═C). ^{1}H-NMR (DMSO-d_6) *δ:* 7.65-7.09 (m, 8*H*, H-Ar), 5.75 (br.s, 2*H*, NH_2, D_2O exchangeable), 5.10 (s, 1*H*, H-pyridine), 4.20 (q, 2*H*, CH_2), and 1.23 (t, 3*H*, CH_3). MS, *m*/*z* (%): 537 ($M^{+\cdot} - 26$, 0.3%).

4.16 | 2,9-Diamino-4,7-bis(4-chlorophenyl)-3-phenyl-4H,7H-pyrano[2′,3′:4,5]thiazolo[3,2-a]pyridine-6,8-dicarbonitrile (14)

A solution of benzyl cyanide (0.02 mol, 2.34 g) in the least amount of dry benzene (10 mL) was stirred at room temperature for 10 minutes. Then solid sodium methoxide (0.23 g of sodium metal in 10 mL of absolute methanol then evaporation till dryness) was added and a solution of **1** (0.01 mol, 4.51 g) in dry benzene (10 mL) was added dropwise within 30 minutes. With stirring to benzyl cyanide solution, the reaction mixture was heated using water bath for 10 minutes, cooled, collected the formed precipitate and recrystallized from mixed benzene/ethanol to give **14**.

Yield (40%), mp 244°C-245°C (benzene/ethanol). Anal. Calcd. For $C_{30}H_{19}Cl_2N_5OS$ (568): C, 63.39; H, 3.37; N, 12.32. Found: C, 63.42; H, 3.40; N, 12.29. FT-IR (KBr) (cm^{-1}): 3344-3213 ($2NH_2$), 2214 (CN), 1637 (C═C). ^{1}H-NMR (DMSO-d_6) δ: 7.95-7.01 (m, 13*H*, H-Ar), 5.09 (br.s, 2*H*, NH_2, D_2O exchangeable), 4.65 (br.s, 2*H*, NH_2, D_2O exchangeable), 4.43 (s, 1*H*, H-pyridine), and 3.90 (s, 1*H*, H-pyrane). MS, *m*/*z* (%): 570 ($M^{+\cdot} + 2$, 10%).

4.17 | 9-Amino-3-chloro-4,7-bis(4-chlorophenyl)-2-oxo-2H,7H-pyrano[2′,3′:4,5]thiazolo[3,2-a]pyridine-6,8-dicarbonitrile (15)

Yield (25%), mp >300°C (dioxane). Anal. Calcd. For $C_{24}H_{11}Cl_3N_4O_2S$ (526): C, 54.83; H, 2.11; N, 10.66.

Found: C, 54.88; H, 2.12; N, 10.70. FT-IR (KBr) (cm^{-1}): broad band at 3417 (NH_2), 2207 (CN), 1716 (C═O), 1633 (C═C). ^{1}H-NMR (DMSO-d_6) δ: 8.55 (br.s, 2*H*, NH_2, D_2O exchangeable), 7.64-7.33 (m, 8*H*, H-Ar), and 4.12 (s, 1*H*, H-pyridine). MS, *m*/*z* (%): 526 ($M^{+\cdot}$, 10%).

4.18 | 9-Amino-4,7-bis(4-chlorophenyl)-2-oxo-2H,7H-pyrano[2′,3′:4,5]thiazolo[3,2-a]pyridine-3,6,8-tricarbonitrile (16)

Yield (42%), mp 254°C-256°C (ethanol). Anal. Calcd. For $C_{25}H_{11}Cl_2N_5O_2S$ (516): C, 58.15; H, 2.15; N, 13.56. Found: C, 58.18; H, 2.13; N, 13.60. FT-IR (KBr) (cm^{-1}): 3330-3204 (NH_2), 2202 (CN), 1721 (C═O), 1641 (C═C). ^{1}H-NMR (DMSO-d_6) δ: 7.86-7.51 (m, 8*H*, H-Ar), 5.88 (br. s, 2*H*, NH_2, D_2O exchangeable), and 4.25 (s, 1*H*, H-pyridine). MS, *m*/*z* (%): 512 ($M^{+\cdot}$ − 4, 0.13%).

4.19 | 5,8-Bis(4-chlorophenyl)-4,9-dioxo-2,11-diphenyl-3,5,9,12-tetrahydro-4H,8H-pyrimidino[3‴,2‴:5″,6″]pyrano[2″,3″:4′,5′]thiazolo[3′,2′:1,6]pyrido[2,3-d]pyrimidine-6-carbonitrile (17)

A mixture of compound **2** (0.01 mol, 5.17 g) and benzoyl chloride (20 mL) was heated under reflux for 24 hours. After evaporation of the solvent, the formed product was recrystallized with petroleum ether (80°C-100°C) to give **17**.

Yield (54%), mp 114°C-116°C, petroleum ether (80°C-100°C). Anal. Calcd. For $C_{39}H_{22}Cl_2N_6O_3S$ (725): C, 64.56; H, 3.06; N, 11.58. Found: C, 64.55; H, 3.09; N, 11.61. FT-IR (KBr) (cm^{-1}): 3305 (2NH), 2220 (CN), 1667 (2C═O), 1602 (C═C). ^{1}H-NMR (DMSO-d_6) δ: 12.91 (br.s, 2*H*, 2NH, D_2O exchangeable), 7.92-7.46 (m, 18*H*, H-Ar), 5.41 (s, 1*H*, H-pyridine), and 4.19 (s, 1*H*, H-pyrane). MS, *m*/*z* (%): 551 ($M^{+\cdot}$ − 173, 3%).

4.20 | 4,9-Diamino-5,8-bis(4-chlorophenyl)-5H,8H-pyrimidino[3‴,4‴:5″,6″]pyrano[2″,3″:4′,5′]thiazolo[3′,2′:1,6]pyrido[2,3-d]pyrimidine-6-carbonitrile (18)

A solution of compound **2** (0.01 mol, 5.17 g) in formamide (20 mL) was heated under reflux for 12 hours. The reaction mixture was poured into ice/water and the formed precipitate was filtered off, dried and recrystallized from ethanol to give **18**.

Yield (36%), mp >300°C (ethanol). Anal. Calcd. For $C_{27}H_{16}Cl_2N_8OS$ (571): C, 56.75; H, 2.82; N, 19.61. Found: C, 56.71; H, 2.85; N, 19.60. FT-IR (KBr) (cm^{-1}): 3418-3219 ($2NH_2$), 2206 (CN), 1630 (C═N). ^{1}H-NMR (DMSO-d_6) δ: 8.15, 7.92 (s, 2*H*, H-pyrimidine), 7.76-7.51 (m, 8*H*, H-Ar), 6.12 (br.s, 4*H*, $2NH_2$, D_2O exchangeable), 4.25 (s, 2*H*, H-pyridine, H-pyrane). MS, *m*/*z* (%): 567 ($M^{+\cdot}$ − 4, 13%).

4.21 | 4,7-Bis(4-chlorophenyl)-2-(([E]-4-nitrobenzylidene)amino)-9-([4-nitro benzylidene]amino)-4H,7H-pyrano[2′,3′:4,5]thiazolo[3,2-a]pyridine-3,6,8-tri carbonitrile (19)

A mixture of compound **2** (0.01 mol, 5.17 g) and *p*-nitro benzaldehyde (0.01 mol and/or 0.02 mol) in glacial acetic acid (30 mL) was heated under reflux for 8 hours. The formed precipitate was filtered off, dried and recrystallized from ethanol to give **19**.

Yield (25%) and/or (60%), mp >300°C (ethanol). Anal. Calcd. For $C_{39}H_{20}Cl_2N_8O_5S$ (783): C, 59.78; H, 2.57; N, 14.30. Found: C, 59.81; H, 2.56; N, 14.35. FT-IR (KBr) (cm^{-1}): 2210 (CN), 1624 (C═N). ^{1}H-NMR (DMSO-d_6) δ: 10.16 (s, 2*H*, H-benzylidenimin), 8.43-7.57 (m, 16*H*, H-Ar), 5.62 (s, 1*H*, H-pyridine), and 5.15 (s, 1*H*, H-pyrane). ^{13}C-NMR (DMSO-d_6) δ: 170.2, 163.5 (2), 155.3, 152.0, 150.3 (2), 143.3, 140.1 (2), 139.6 (2), 131.4 (2), 130.4 (4), 128.9 (4), 127.5 (4), 124.0 (4), 118.3, 117.1 (2), 84.1, 76.3, 76.1, 70.5, 41.2, 38.2. MS, *m*/*z* (%): 784 ($M^{+\cdot}$ + 1, 24%).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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