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APPLICATION OF MANNICH AND MICHAEL REACTIONS IN SYNTHESIS OF PYRIDOPYRIMIDO[2,1-*b*][1,3,5]THIADIAZINONES AND PYRIDOPYRIMIDO[2,1-*b*][1,3]-THIAZINONES AS ANTICANCER AGENTS

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Abstract – A new series of pyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazinones were prepared by aminomethylation of pyridopyrimidinethione with a variety of primary aromatic amines and formaldehyde solution (37%) through Mannich reaction. Also, another series of pyrido[2',3:4,5]pyrimido[2,1-*b*][1,3]thiazinones were synthesized by Micheal addition reaction of pyridopyrimidinethione to the activated double bond of a number of arylidene malononitrile and ethyl 3-aryl-2-cyanopropenoate. The mechanism of formation of the synthesized compounds was discussed and the assigned structure was established *via* microanalysis and spectral data (IR, ^{1}HNMR and Mass). In addition, the antitumor activities of the synthesized compounds were investigated in comparison with the well-known anticancer standard drugs doxorubcin and Imatinib using MTT assay. The results revealed that the tested compounds showed high variation in the inhibitory growth rates and activities against the tested tumor cell lines in a concentration dependent manner. The highest activity was measured for compound **4g** against human hepatocellular carcinoma (HepG2) cells and compound **4i** in case of breast carcinoma (MCF-7) cells compared with reference drug imatinib. These results revealed that these compounds exhibited promising antitumor activities.

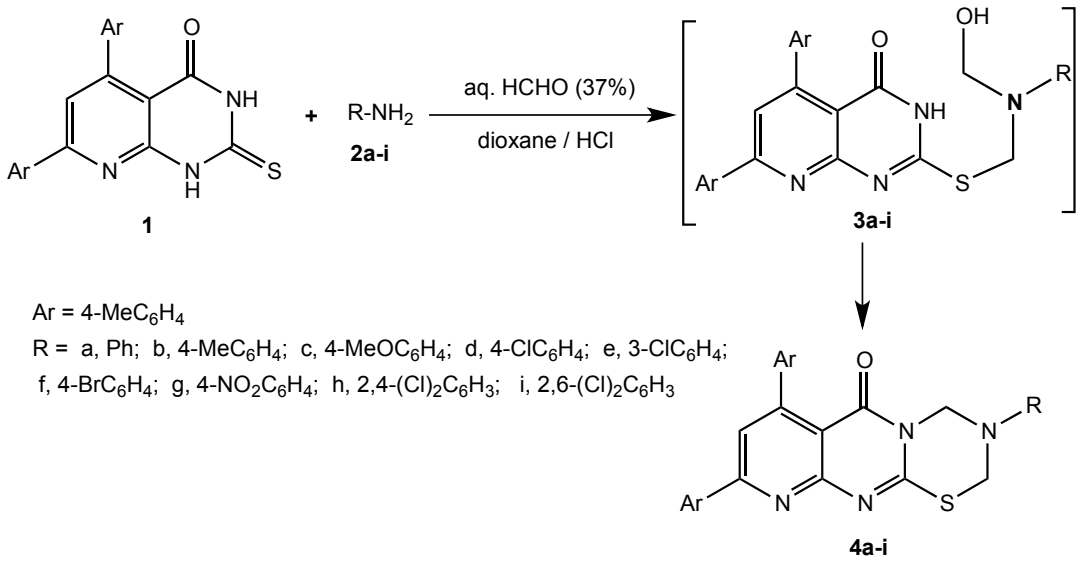
INTRODUCTION

Pyrimidine and fused heterocyclic pyrimidine derivatives have received significant attention over the past few years owing to their therapeutic and pharmacological properties.[1-5] For example, pyridopyrimidines

have exhibited promising biological and pharmacological activities such as antibacterial,[6] antifolate,[7] tyrosine kinase activity,[8] anti-inflammatory, analgesic,[9] antimicrobial,[10] calcium channel antagonist,[11] antileishmania,[12] anti-convulsant,[13] tuberculostatic,[14] diuretic and potassium-sparing,[15] and anti-aggressive activities.[16] On the other hand, fused pyridopyrimidines are also reported to have a wide range of biological activities.[17-20] Based on all these findings, we intended to pyridopyrimidines and their fused ring systems. In view of all these facts mentioned above and as part of our program to search for potentially bioactive new agents,[21-30] we report herein the synthesis of novel pyridopyrimido[2,1-*b*][1,3]thiazinone and pyridopyrimido[2,1-*b*][1,3,5]thiadiazinone derivatives. Also, the anticancer activities of the target compounds were evaluated.

RESULTS AND DISCUSSION

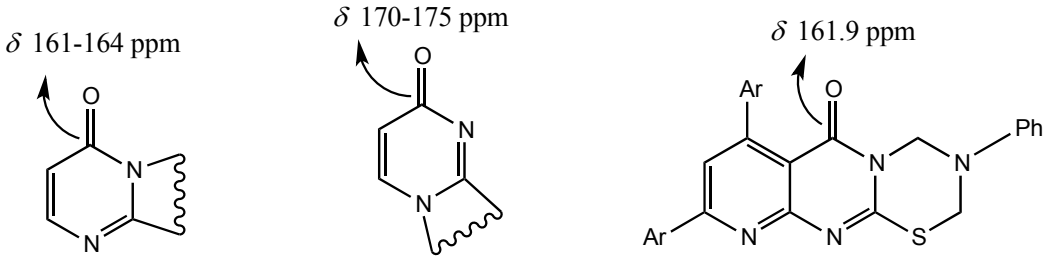
The starting compound, 2-thioxo-5,7-di-*p*-tolyl-2,3-dihydropyrido[2,3-d]pyrimidin-4(1*H*)-one (**1**) was prepared *via* reacting 6-amino-2-thiouracil and 1,3-di-p-tolylprop-2-en-1-one in DMF according to the procedure reported by Quiroga *et al*.[31] (Scheme 1). The structure of the compound **1** was confirmed by elemental and spectral data. The reaction of thione **1** with each of the substituted anilines **2a-i** and excess aqueous formaldehyde solution (37%) in dioxane in the presence of a few drops of conc. hydrochloric acid afforded a single product as evidenced by TLC analysis of the crude product. However, the elemental analysis and mass spectral data of the isolated products were consistent with compound **4** (Scheme 1).



Scheme 1. Synthesis of pyridopyrimidothiadiazinone derivatives **4a-i**

Literature reports[32-36] have shown that the cyclization of S-alkylated pyrimidinones occure at N-atom which adjacent to C=O group rather than the other N-atom, based on ^{13}C NMR data. For example, ^{13}C NMR spectral data of compound **4a** revealed carbonyl carbon signals of the pyrimidinone at 161.9 ppm,

suggesting that N-atom adjacent to C=O is sp^3-hybridized which is different from C=O adjacent to a sp^2-hybridized nitrogen that usually appears at 170-175 ppm.[36] Accordingly, the structure of compound **4b** existed in one form namely, **A** rather than **B**. Recently, Fares *et al.* gave an absolute confirmation that the cyclization occurred at N-atom which adjacent to C=O group based on single-crystal X-ray analysis,[37] therefore the structures of the products **4a-i** have been formulated as the linear isomers **A** not the isomeric angular isomers **B** (Figure 1).

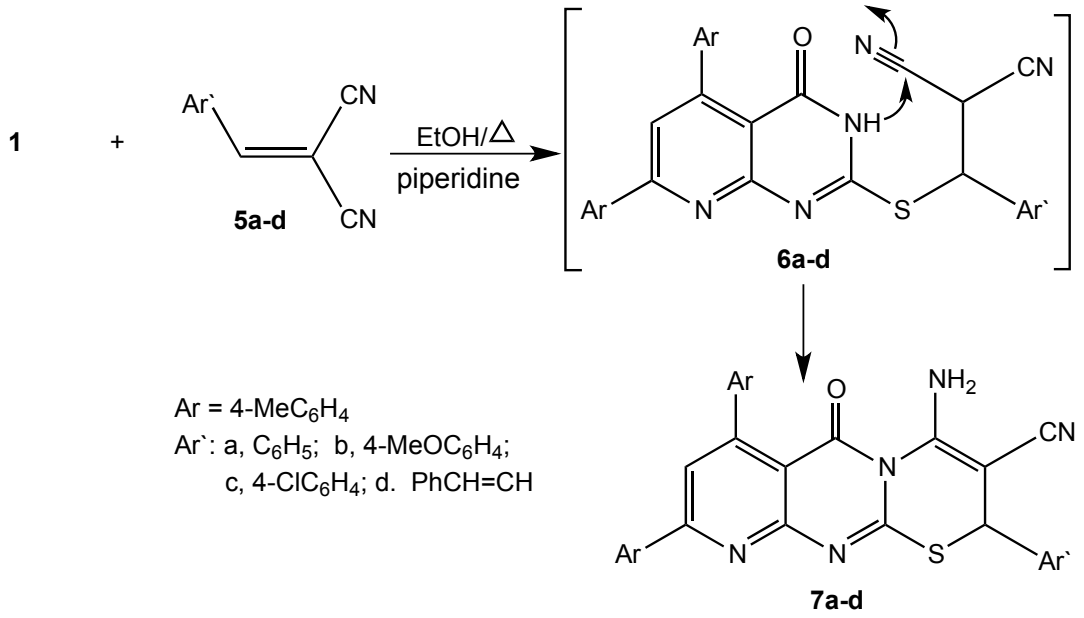


A **B** **4a**

Figure 1. The strategic structures of the products **4a-i**

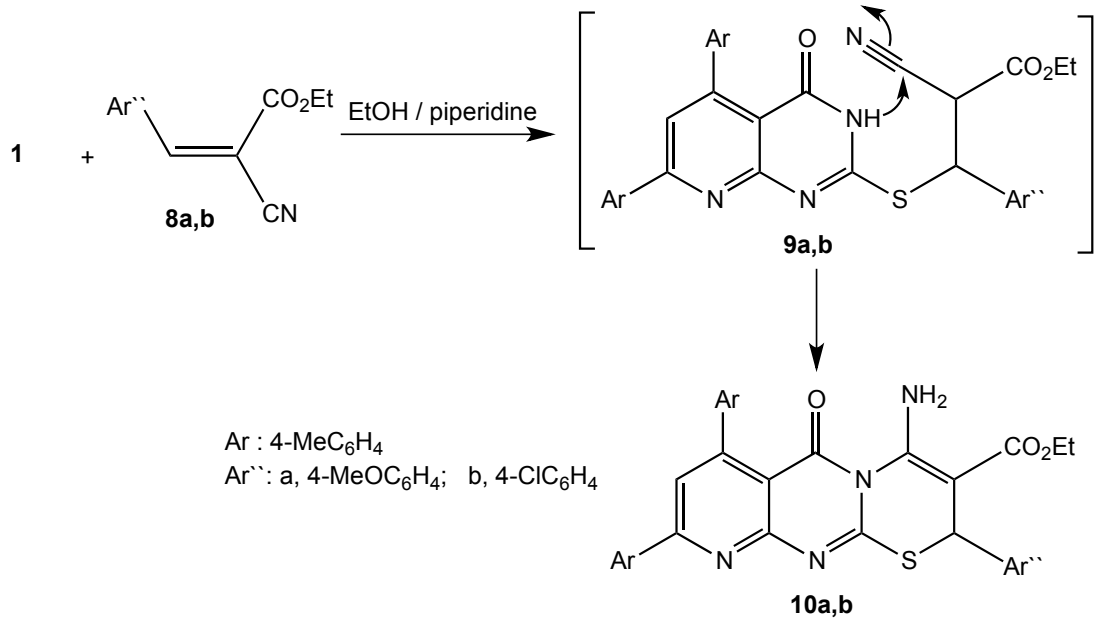
The formation of products **4** from reaction of pyridopyrimidinethione **1** with amines and formaldehyde solution indicated that the intermediate **3** underwent *in situ* cyclization as soon as they are formed *via* elimination of water molecule (Scheme 1).

Another series of fused pyrimidothiazinones were prepared based on application of Micheal addition reaction. Thus, reaction of pyridopyrimidinethione **1** with each of the appropriate arylidene malononitrile **5a-d** in ethanol under reflux in the presence of a catalytic amount of piperidine yielded the pyridopyrimidothiazinones **7a-e**. The structure of the latter products was evidenced by microanalysis and spectral (IR, ^{1}H NMR, Mass) data. For example, the IR spectra of the latter products revealed in each case three absorption bands near ν 1650, 2212, 3198, 3388 cm^{-1} attributed to the carbonyl, nitrile and the amino groups. Also, ^{1}H NMR displayed in each case two singlet signals near δ 4.14, 8.73, assigned for the –SCH-Ar proton of the thiazine ring and amino group, in addition to the expected signals characteristic for the aryl protons (see experimental). The mass spectra of products **7** revealed in each case a molecular ion peak which consistent with the molecular formula of the assigned structure. A reasonable mechanism was outlined in Scheme 2 to account for the formation of products **7**. It was suggested that the reaction of pyridopyrimidinethione **1** with arylidene malononitrile proceeded by initial Micheal addition of the thiol group to the activated double bond of compound **5** to give the non-isolable intermediate **6** which underwent tandem intramolecular cyclization and tautomerism to give the final products **7** (Scheme 2).



Scheme 2. Synthesis of pyridopyrimidothiazinone derivatives **7a-d**

Similarly, pyridopyrimidothiazinone derivatives **10a,b** were prepared by Michael addition reaction of thione **1** with each of ethyl 3-aryl-2-cyanopropenoate **8a,b** under the same reaction conditions (Scheme 3). The structure assigned for the products **10** was confirmed based on elemental analysis and spectroscopic data. For example, the IR spectra of products **10** revealed in each case two absorption bands in the region ν 1634, 1671cm^{-1} due to the carbonyl groups of pyrimidinone ring and the ester group, in addition to the characteristic absorption band at ύ 3211, 3417 cm^{-1} attributed to the amino group. Further evidence for structure **10** was achieved based on the ^{1}H NMR and mass spectral data.



Scheme 3. Synthesis of pyridopyrimidothiazine derivatives **10a,b**

Thus, ^{1}H NMR spectra revealed the characteristic signals of the ester protons at δ 1.36, 4.31 ppm, two singlet signals at δ 4.24, 8.72 ppm assigned for the –SCH-Ar and $-NH_2$ protons, in addition to the expected signals assigned for the aromatic protons. The mass spectra of **10** revealed in each case a molecular ion peak which is consistent with the assigned structure. The mechanism of formation of products **10** proceeds by the same way as suggested for products **7** (Scheme 3).

BIOLOGICAL SCEENING

Cytotoxic Activity

The *in vitro* growth inhibitory rates (%) and inhibitory growth activity (as measured by IC_{50}) of the synthesized compounds were investigated in comparison with the well-known anticancer standard drugs doxorubicin and Imatinib (2-substituted aminopyrimidine derivative; Gleevec®), using MTT viability assay. However, compound **10a** was almost inactive; the results revealed that the tested compounds showed high variation in the inhibitory growth rates and activities against the tested tumor cell lines in a concentration dependent manner. The highest activity against human hepatocellular carcinoma (HepG2) cell line was measured for compound **4g** with IC_{50} value 3.0 x 10^{-2} µM, compared with reference drug imatinib followed by compounds **7b, 4i, 4d, 4e, 4c, 10b, 7a, 4b, 4h, 4f, 7c,** and **4a**, respectively.

The tested compounds also showed higher inhibitory effects against human hepatocellular carcinoma (HepG2) than human breast carcinoma (MCF-7) cell lines. The highest detected inhibitory activities against human breast carcinoma (MCF-7) cell line was measured for compound **4i** compared with reference drug imatinib followed by **7b, 4c, 4g, 4d, 4e, 10b, 7a, 4b, 4h, 4f**, **7c** and **4a**, respectively (Table 1). The difference between inhibitory activities of all compounds with different concentrations was statistically significant $P < 0.001$.

Table 1. The antitumor activities of the tested compounds compared with reference standard drugs evaluated using MTT assay on breast and liver cancer cell lines

Tested compounds	IC_{50} values (µM) against tumor cell lines	
	MCF-7	HepG2
4a	42.0 x 10^{-2}	37.9 x 10^{-2}
4b	17.4 x 10^{-2}	9.6 x 10^{-2}
4c	6.2 x 10^{-2}	1.9 x 10^{-2}
4d	4.3 x 10^{-2}	2.3 x 10^{-2}
4e	4.7 x 10^{-2}	3.3 x 10^{-2}
4f	33.7 x 10^{-2}	26.6 x 10^{-2}

4g	3.0×10^{-2}	2.0×10^{-2}
4h	26.7×10^{-2}	12.6×10^{-2}
4i	3.7×10^{-2}	1.5×10^{-2}
7a	9.5×10^{-2}	4.2×10^{-2}
7b	3.1×10^{-2}	1.8×10^{-2}
7c	36.5×10^{-2}	33.6×10^{-2}
10a	$>169.4 \times 10^{-2}$	$>169.4 \times 10^{-2}$
10b	6.84×10^{-2}	3.5×10^{-2}
Doxorubicin	0.1472×10^{-2}	0.184×10^{-2}
Imatinib	4.96×10^{-2}	4.94×10^{-2}

CONCLUSIONS

Pyridopyrimidothiadiazines were prepared by adopting simple synthetic strategy depending on aminomethylation of pyridopyrimidinethione through Mannich reaction. Another series of pyridopyrimidothiazines were easily synthesized by application of Micheal addition reaction of pyridopyrimidinethione with a number of activated nitrile compounds. The structure assigned for all the products was established *via* elemental and specrtroscopic techniques. Also, the mechanisms account for formation of the products was discussed. The evaluation of the antitumor activity of the synthesized compounds revealed that the highest activity was measured for compound **7b** against human hepatocellular carcinoma (HepG2) cells and compound **4g** in case of breast carcinoma (MCF-7) cells.

EXPERIMENTAL

Melting points were recorded on a Gallenkamp electrothermal apparatus. Infrared spectra (KBr) were determined on a Pye Unicam SP-3000 infrared spectrophotometer. ^{1}H NMR was determined on a Varian Gemini 300 spectrometer (300 MHz) in DMSO-d_6 with TMS as internal standard. Mass spectra were recorded on a GCMS-QP 1000 EX Shimadzu spectrometer. Elemental analyses were carried out at the Microanalytical center, University of Cairo, Giza, Egypt. The biological evaluation of the products was carried out at the Regional Center for Mycology and Biotechnology at Al-Azhar University, Cairo, Egypt.

Synthesis of 2-thioxo-5,7-di-*p*-tolyl-2,3-dihydropyrido[2,3-*d*]pyrimidin-4(1*H*)-one (**1**).

A mixture of 1,3-di-*p*-tolylprop-2-en-1-one (2.36 g, 10 mmol) and 6-amino-2-thiouracil (1.43 g, 10 mmol) in dry DMF (30 mL) was refluxed for 15 h. The reaction mixture was cooled and the solid formed was filtered, dried and crystallized from DMF. Yellowish-white solid, mp 332-334 ºC; yield 72%; IR (KBr): ν 3432, 3360 (2NH), 1673 (C=O), 1603 (C=N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.28 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 7.21-7.75 (m, 8H, Ar-H), 7.90 (s, 1H, pyridine-H5), 11.03, 11.64 (2s, D_2O

exchangeable, 2H, 2NH); MS (70 eV): *m*/*z* 359 (M^+, 35), 270 (100), 193 (51), 104 (48), 77 (73). Anal. Calcd for $C_{21}H_{17}N_3OS$ (359.44): C, 70.17; H, 4.77; N, 11.69%. Found: C, 70.11; H, 4.64; N, 11.45%.

Synthesis of 3,7,9-triaryl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-ones 4a-i. General procedure: A mixture of thione **1** (0.359 g, 1 mmol), 37% formaldehyde solution (2 mL) and the appropriate aniline derivative **2a-i** (1 mmol) in dioxane (20 mL) in the presence of few drops of HCl was stirred at room temperature for 4-8 h (monitored by TLC). The solid that precipitated was filtered off, washed with water, dried and finally crystallized from dioxane or EtOH to give the respective products **4a-i**.

3-Phenyl-7,9-di-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4a).Yellow solid; yield 74%; mp 135-137 °C (dioxane); IR (KBr): ν 1594 (C=N), 1654 (C=O), 2944, 3026 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.25 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 5.44 (s, 2H, CH_2), 5.79 (s, 2H, CH_2), 6.87-8.07 (m, 13H, Ar-H), 7.84 (s, 1H, pyridine-H5); ^{13}C NMR (300 MHz, DMSO-d_6): δ 15.2, 15.6 (CH_3), 53.1, 60.9 (CH_2), 115.0, 118.5, 121.5, 124.2, 125.0, 126.9, 127.1, 128.2, 128.9, 129.4, 130.0, 132.1, 134.9, 138.0, 138.7, 139.7, 142.1, 147.5 (Ar-C), 161.9 (C=O) ppm; MS (70 eV): *m*/*z* = 476 (M^+, 31), 461(23), 373 (17), 359 (100), 221 (24), 119 (48), 105 (39), 77 (69). Anal. Calcd for $C_{29}H_{24}N_4OS$ (476.17): C, 73.08; H, 5.08; N, 11.76. Found: C, 73.01; H, 5.03; N, 11.52%.

3,7,9-Tri-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4b). Yellowish-brown solid; yield 77%; mp 140-142 °C (dioxane); IR (KBr): ν 1594 (C=N), 1655 (C=O), 2916, 3026 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.22 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 5.76 (s, 2H, CH_2), 6.85-8.07 (m, 12H, Ar-H), 7.83 (s, 1H, pyridine-H5); MS (70 eV): *m*/*z* 490 (M^+, 26), 461 (73), 359 (94), 221 (38), 119 (100), 91 (97), 65 (47). Anal. Calcd for $C_{30}H_{26}N_4OS$ (490.18): C, 73.44; H, 5.34; N, 11.42. Found: C, 73.29; H, 5.27; N, 11.29%.

3-(4-Methoxyphenyl)-7,9-di-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4c). Pale green solid; yield 70%; mp 120-122 °C (EtOH); IR (KBr): ν 1592 (C=N), 1652 (C=O), 2912, 2971, 3028 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 3.76 (s, 3H, OCH_3), 5.42 (s, 2H, CH_2), 5.77 (s, 2H, CH_2), 7.26-8.07 (m, 12H, Ar-H), 7.84 (s, 1H, pyridine-H5); MS (70 eV): *m*/*z* 506 (M^+, 37), 469 (52), 358 (62), 221 (38), 119 (84), 80 (71), 64 (100). Anal. Calcd for $C_{30}H_{26}N_4O_2S$ (506.18): C, 71.12; H, 5.17; N, 11.06. Found: C, 71.06; H, 5.11; N, 10.85%.

3-(4-Chlorophenyl)-7,9-di-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4d). Brown solid; yield 76%; mp 133-135 °C (dioxane); IR (KBr): ν 1594 (C=N), 1655 (C=O), 2916, 3026 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 5.17 (s, 2H, CH_2), 5.74 (s, 2H, CH_2), 6.62-8.07 (m, 12H, Ar-H), 7.85 (s, 1H, pyridine-H5); MS (70 eV):

m/*z* 512 (M^++2 , 11), 510 (M^+ , 37), 461 (42), 359 (100), 275 (29), 119 (68), 91 (73), 64 (41). Anal. Calcd for $C_{29}H_{23}ClN_4OS$ (510.13): C, 68.16; H, 4.54; N, 10.96. Found: C, 68.10; H, 4.48; N, 10.79%.

3-(3-Chlorophenyl)-7,9-di-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4e). Orange solid; yield 72%; mp 155-157 °C (EtOH); IR (KBr): ν 1593 (C=N), 1663 (C=O), 2961, 3028 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 5.41 (s, 2H, CH_2), 5.75 (s, 2H, CH_2), 7.23-8.07 (m, 12H, Ar-H), 7.84 (s, 1H, pyridine-H5); MS (70 eV): *m*/*z* 512 (M^++2 , 6), 510 (M^+ , 21), 482 (63), 359 (54), 221 (41), 119 (47), 91 (66), 64 (100). Anal. Calcd for $C_{29}H_{23}ClN_4OS$ (510.13): C, 68.16; H, 4.54; N, 10.96. Found: C, 68.04; H, 4.46; N, 10.78%.

3-(4-Bromophenyl)-7,9-di-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4f). Yellow solid; yield 76%; mp 133-135 °C (dioxane); IR (KBr): ν 1593 (C=N), 1655 (C=O), 2916, 2944, 3026 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.32 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 5.41 (s, 2H, CH_2), 5.68 (s, 2H, CH_2), 6.97-8.06 (m, 12H, Ar-H), 7.84 (s, 1H, pyridine-H5); MS (70 eV): *m*/*z* 556 (M^++2 , 11), 554 (M^+ , 13), 372 (33), 359 (100), 221 (57), 119 (75), 91 (98), 65 (52). Anal. Calcd for $C_{29}H_{23}BrN_4OS$ (554.08): C, 62.70; H, 4.17; N, 10.09. Found: C, 62.59; H, 4.05; N, 10.01%.

3-(4-Nitrophenyl)-7,9-di-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4g). Yellow solid; yield 77%; mp 115-117 °C (dioxane); IR (KBr): ν 1594 (C=N), 1653 (C=O), 2914, 3027 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 5.27 (s, 2H, CH_2), 5.62 (s, 2H, CH_2), 6.76-8.15 (m, 12H, Ar-H), 7.89 (s, 1H, pyridine-H5); MS (70 eV): *m*/*z* 521 (M^+ , 52), 481 (53), 359 (94), 221 (33), 119 (65), 91 (69), 64 (100). Anal. Calcd for $C_{29}H_{23}N_5O_3S$ (521.15): C, 66.78; H, 4.44; N, 13.43. Found: C, 66.58; H, 4.31; N, 13.28%.

3-(2,4-Dichlorophenyl)-7,9-di-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4h). Yellow solid; yield 75%; mp 150-152 °C (dioxane); IR (KBr): ν 1592 (C=N), 1652 (C=O), 2912, 2969, 3028 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 5.69 (s, 2H, CH_2), 7.26-8.07 (m, 11H, Ar-H), 7.84 (s, 1H, pyridine-H5); MS (70 eV): *m*/*z* 544 (M^+ , 17), 447 (31), 207 (25), 105 (47), 80 (100), 64 (60). Anal. Calcd for $C_{29}H_{22}Cl_2N_4OS$ (544.09): C, 63.85; H, 4.07; N, 10.27. Found: C, 63.74; H, 4.12; N, 10.17%.

3-(2,6-Dichlorophenyl)-7,9-di-*p*-tolyl-3,4-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3,5]thiadiazin-6(2*H*)-one (4i). Yellow solid; yield 69%; mp 127-129 °C (EtOH); IR (KBr): ν 1592 (C=N), 1653 (C=O), 2912, 2940, 3028 (C-H) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.35 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 5.43 (s, 2H, CH_2), 5.74 (s, 2H, CH_2), 7.26-8.09 (m, 11H, Ar-H), 7.86 (s, 1H, pyridine-H5); MS (70 eV): *m*/*z* 544 (M^+ , 13), 445 (42), 371 (37), 221 (47), 119 (63), 80 (100), 64 (52). Anal. Calcd for $C_{29}H_{22}Cl_2N_4OS$ (544.09): C, 63.85; H, 4.07; N, 10.27. Found: C, 63.64; H, 4.02; N, 10.13%.

Synthesis of pyridopyrimidothiazinone derivatives 7a-d

General procedure: To a solution of thione **1** (0.359 g, 1 mmol) and the appropriate (arylidene)malononitrile **5a-d** (1 mmol) in 20 mL of EtOH was added 0.5 mL of piperidine and the mixture was refluxed for 8 h. The solid precipitated after cooling was filtered off, washed with water, dried and finally crystallized from EtOH to give products **7a-d.** The physical and spectral data of products **7a-d** are depicted as follows.

4-Amino-6-oxo-2-phenyl-7,9-di-*p*-tolyl-2,6-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3]thiazine-3-carbonitrile (7a). Brown solid; yield 69%; mp 178-180 ºC; IR (KBr): ν 1593 (C=N),1630 (C=O), 2188 (CN), 2942, 3034 (C-H), 3200, 3336 (NH_2) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.37 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 4.01 (s, 1H, CH), 7.05-8.06 (m, 13H, Ar-H), 7.86 (s, 1H, pyridine-H5), 8.71 (s, 2H, NH_2, D_2O exchangeable); MS (70 eV): *m*/*z* 513 (M^+, 7), 384 (25), 302 (81), 250 (98), 91 (18), 80 (88), 64 (100). Anal. Calcd for $C_{31}H_{23}N_5OS$ (513.16): C, 72.49; H, 4.51; N, 13.64. Found: C, 72.34; H, 4.39; N, 13.47%.

4-Amino-2-(4-methoxyphenyl)-6-oxo-7,9-di-*p*-tolyl-2,6-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*]-[1,3]thiazine-3-carbonitrile (7b). Brown solid; yield 66%; mp 167-169 ºC; IR (KBr): ν 1600 (C=N),1650 (C=O), 2212 (CN), 2938, 3026 (C-H), 3198, 3388 (NH_2) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.37 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 4.14 (s, 1H, CH), 7.05-8.15 (m, 12H, Ar-H), 7.76 (s, 1H, pyridine-H5), 8.73 (s, 2H, NH_2, D_2O exchangeable); MS (70 eV): *m*/*z* 543 (M^+, 7), 373 (55), 235 (36), 221 (95), 119 (59), 80 (80), 64 (100). Anal. Calcd for $C_{32}H_{25}N_5O_2S$ (543.17): C, 70.70; H, 4.64; N, 12.88. Found: C, 70.53; H, 4.57; N, 12.69%.

4-Amino-2-(4-chlorophenyl)-6-oxo-7,9-di-*p*-tolyl-2,6-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*]-[1,3]thiazine-3-carbonitrile (7c). Brown solid; yield 67%; mp 230-232 ºC; IR (KBr): ν 1600 (C=N),1649 (C=O), 2207 (CN), 2931, 3059 (C-H), 3231, 3410 (NH_2) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.28 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 4.14 (s, 1H, CH), 7.08-8.14 (m, 12H, Ar-H), 7.56 (s, 1H, pyridine-H5), 8.74 (s, 2H, NH_2, D_2O exchangeable); MS (70 eV): *m*/*z* 549 (M^++2 , 9), 547 (M^+ , 20), 373 (55), 342 (97), 230 (50), 164 (56), 80 (43), 64 (100). Anal. Calcd for $C_{31}H_{22}ClN_5OS$ (547.12): C, 67.94; H, 4.05; N, 12.78. Found: C, 67.82; H, 4.00; N, 12.64%.

4-Amino-6-oxo-2-(2-phenylethenyl)-7,9-di-*p*-tolyl-2,6-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*]-[1,3]thiazine-3-carbonitrile (7d). Brown solid; yield 67%; mp 230-232 ºC; IR (KBr): ν 1601 (C=N),1630 (C=O), 2215 (CN), 2936, 3032 (C-H), 3211, 3426 (NH_2) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.31 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 4.14 (s, 1H, CH), 6.37 (m, 1H, -<u>CH</u>=CH-Ph), 6.71 (d, *J* = 16 *Hz*, 1H, -CH=<u>CH</u>-Ph), 7.05-8.07 (m, 13H, Ar-H), 7.68 (s, 1H, pyridine-H5), 8.70 (s, 2H, NH_2, D_2O exchangeable); MS (70 eV): *m*/*z* 539 (M^+ , 16), 473 (46), 361 (72), 230 (100), 119 (38), 80 (52), 64 (80). Anal. Calcd for $C_{33}H_{25}N_5OS$ (539.18): C, 73.45; H, 4.67; N, 12.98. Found: C, 73.29; H, 4.61; N, 12.86%.

Synthesis of pyridopyrimidothiazinone derivatives 10a,b

General procedure: To a solution of thione **1** (0.359 g, 1 mmol) and ethyl 3-aryl-2-cyanopropenoate **8a,b** (1 mmol) in 20 EtOH was added 0.5 mL of piperidine and the mixture was warmed for 8 hr. The solid that precipitated was filtered off, washed with water, dried and finally crystallized from DMF to give the respective products **10a,b.**

Ethyl 4-amino-2-(4-methoxyphenyl)-6-oxo-7,9-di-*p*-tolyl-2,6-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3]thiazine-3-carboxylate (10a). Yellow solid; yield 69%; mp 105-107 ºC; IR (KBr): ν 1590 (C=N), 1634, 1671 (2 C=O), 2938, 3061 (C-H), 3211, 3417 (NH_2) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 1.36 (t, J = 6.9Hz, 3H, CH_3CH_2), 2.28 (s, 3H, CH_3), 2.37 (s, 3H, CH_3), 3.82 (s, 3H, OCH_3), 4.24 (s, 1H, CH), 4.31 (q, J = 6.9Hz, 2H, CH_2CH_3), 7.08-8.02 (m, 12H, Ar-H), 7.69 (s, 1H, pyridine-H5), 8.72 (s, 2H, NH_2, D_2O exchangeable); MS (70 eV): m/z 590 (M^+, 52), 554 (46), 426 (70), 310 (92), 143 (44), 91 (38), 64 (100). Anal. Calcd for $C_{34}H_{30}N_4O_4S$ (590.20): C, 69.13; H, 5.12; N, 9.48. Found: C, 69.05; H, 5.07; N, 9.33%.

Ethyl 4-amino-2-(4-chlorophenyl)-6-oxo-7,9-di-*p*-tolyl-2,6-dihydropyrido[2',3':4,5]pyrimido[2,1-*b*][1,3]thiazine-3-carboxylate (10b). Yellow solid; yield 72%; mp 180-182 ºC; IR (KBr): ν 1596 (C=N), 1634, 1678 (2 C=O), 2945, 3061 (C-H), 3211, 3433 (NH_2) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 1.38 (t, J = 6.9Hz, 3H, CH_3CH_2), 2.31 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 4.27 (s, 1H, CH), 4.38 (q, J = 6.9Hz, 2H, CH_2CH_3), 7.22-8.12 (m, 12H, Ar-H), 7.72 (s, 1H, pyridine-H5), 8.78 (s, 2H, NH_2, D_2O exchangeable); MS (70 eV): m/z 597 (M^++2, 23), 595 (M^+, 63), 502 (80), 424 (71), 330 (12), 125 (24), 84 (100). Anal. Calcd for $C_{33}H_{27}ClN_4O_3S$ (595.11): C, 66.60; H, 4.57; N, 9.41. Found: C, 66.47; H, 4.52; N, 9.36%.

Antitumor activity assay

The tested human carcinoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 1% L-glutamine, and 50µg/mL gentamycin. The cells were maintained at 37 ºC in a humidified atmosphere with 5% CO_2 incubator (Shel lab 2406, USA) and were sub-cultured two to three times a week. For antitumor assays, the tumor cell lines were suspended in medium at concentration $5x10^4$ cell/well in Corning® 96-well tissue culture plates, then incubated for 24 h. The tested compounds were then added into 96-well plates (six replicates) to achieve eight concentrations for each compound (started from 200 to 1.56 µg/mL). Six vehicle controls with media or 0.1% DMSO were run for each 96 well plate as a control. After incubating for 24 h, the numbers of viable cells were determined by the MTT assay. Briefly, the media was removed from the 96 well plate and replaced with 100 µL of fresh culture RPMI 1640 medium without phenol red then 10 µL of the 12 mM MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Sigma) stock solution (5 mg of MTT in 1 mL of PBS) to each well including the untreated controls. The 96 well plates were then incubated at 37 °C and 5% CO_2

for 4 hours. An 85 µL aliquot of the media was removed from the wells, and 50 µL of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37 °C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [1-(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA) [38, 39].

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