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THE SYNTHESIS OF BIOLOGICALLY ACTIVE PYRAZOLO[3,4-*b*]PYRIDINE AND PYRIDO[2,3-*d*]PYRIMIDINE DERIVATIVES

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ABSTRACT. In this study, firstly, benzylidene derivatives were obtained by Knoevenagel condensation using various aryl aldehydes and malononitrile in the presence of ethanol and then these differently substituted benzylidene compounds were substituted with 5-amino-3-methyl-1-phenylpyrazole and 6-amino-1-amino-1-phenylpyrazole, respectively, 3-dimethyluracil and ytterbium(III) trifluoromethane-sulfonate [$Yb(OTf)_3$] or acetic acid catalyzed pyrazolo[3,4-*b*]pyridine and pyrido[2,3-*d*]pyrimidine derivatives were synthesized (**5a-5i**), and the structures of these compounds, which were purified by different methods, were elucidated by spectroscopic methods such as FTIR, ^{1}H-NMR, ^{13}C-NMR and GS-MS. In our study, compounds **3a**, **5a** and **5c** were synthesized for the first time. In addition, $Yb(OTf)_3$, one of the metal catalysts considered environmentally friendly catalysts, was used in this research. The genotoxic and antigenotoxic properties of the synthesized compounds were investigated in vitro using Ames Salmonella/microsome mutagenicity assay in the concentration range of 0.2-1.0 mM/plate. The results revealed that none of the compounds were mutagenic on three different Salmonella typhimurium strains up to the highest tested concentration. Moreover, in our study, **5a**, **5e**, **5f** and **5h** showed significant antigenotoxic effects ranging from moderate to strong against mutagen-induced DNA damage at relatively higher doses.

KEY WORDS: Prido[2,3-*d*]pyrimidine, Pyrazolo[3,4-*b*]pyridine, Heterocyclic compounds, Benzylidene derivatives

INTRODUCTION

Today, most compounds we encounter as active pharmaceutical ingredients consist of heterocyclic structures containing nitrogen atoms. It is known that these structures, which contain nitrogen in their rings, show various pharmacological and biological activities. These structures, which have activities such as antimalarial, dihydrofolate reductase inhibition, anticancer, anti-hypertensive, anti-inflammatory, antibacterial, anti-leishmanial, antiviral, anti-fungal and cytotoxic, are in the category of important compounds, and researchers are developing various methods for the synthesis of these compounds and their derivatives [1].

In addition, these compounds are used in the pharmaceutical industry and in the chemical agriculture and cosmetics industry; they are also used to reduce wrinkles, strengthen hair follicles ensure healthier growth, and prevent grey hair formation [2].

Recently, these compounds have been synthesized by trying different catalysts and reaction methods such as ionic liquid, PTSA (*p*-toluene sulfonic acid), hydrogen phosphate, SBA-15 (Santa Barbara Amorphous-15), nanoparticles, triflates, L-pyrroline, copper salts, especially the multi-component one-pot method is intended [3-12]. Although these studies found in the literature aim to support green chemistry by using no or small amounts of solvents and using a minimum number of recycled catalysts, it has been observed that there are various drawbacks such as failure to meet the desired conditions and the formation of by-products. The use of triflate as a catalyst in pyrazolo[3,4-*b*]pyridine syntheses was not found in the literature research.

In the first step of this study, benzylidene derivatives were synthesized using various substituted aromatic aldehydes and malononitrile as starting materials under a magnetic stirrer

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and reflux. In the next step, pyridine and pyrimidine derivative compounds were synthesized by reacting these compounds with both 6-amino-1,3-dimethyluracil and 5-amino-3-methyl-1-phenylpyrazole in the presence of ethanol/AcOH and using $Yb(OTf)_3$ catalyst.

To reduce environmental damage and support green chemistry in syntheses, $Yb(OTf)_3$, which is not available in literature, was chosen as the catalyst, and ethyl alcohol was selected as the solvent.

EXPERIMENTAL

Chemicals, reagents, apparatus, and equipment

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and used without any purification. The progress of the reactions was monitored by thin layer chromatography (TLC). In TLC, "Merck 5554" silica gel plates with fluorescent indicator and "Camag (254/366nm)" UV lamp were used (Muttenz, Switzerland). The synthesized products were purified and characterized. Infrared spectra were analysed by "Perkin-Elmer, FT-IR" spectrophotometer (Shelton, USA) titled ATR in Yıldız Technical University Instrumental Analysis Laboratory. Nuclear magnetic resonance spectra (^1H, ^{13}C NMR) were taken according to TMS standards in chloro-form-d($CDCl_3$) and DMSO-d_6 by using 'Bruker AVANCE 500 MHz NMR' instrument (Massachusetts, ABD) in Balıkesir University Science and Technology Application and Research Centre. Mass spectra were analysed by 'Agilent Technologies, 6530 model Q-TOF-LC-MS/MS (California, USA) at Yıldız Technical University Central Laboratory (Turkey). The melting point of the purified products was determined using a Gallenkamp 4947 (Cambridge, England) branded device. Solvents were recovered with a "Heidolph brand RV Laborata 4000 model" rotary evaporator (Schwabach, Germany).

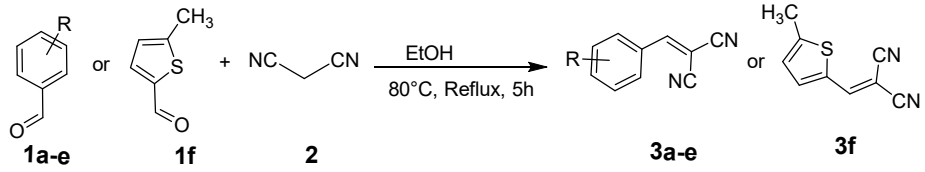
General procedure

*4-Hydroxy-3-methoxy-5-nitrophenyl) methylidenepropandinitrile (compound **3a**)*

A mixture of [(4-hydroxy-3-methoxy-5-nitrophenyl) methylidene] propanedinitrile (compound **3a**), (1 mmol), and 6-amino-1,3-dimethyl uracil (1 mmol) dissolved in 1.5 mL of ethyl alcohol with acetic acid (0,1 mL) and was stirred and boiled under reflux for about 8 hours. The solid product formed from TLC controls was first crystallized, and then purified by two-dimensional thin-layer chromatography, and spectroscopic methods elucidated its structures.

Synthesis of benzylidene malononitrile compounds

A mixture of variously substituted benzaldehyde (or heteroaromatic aldehyde) (2 mmol) and malononitrile (2 mmol) was added to a round-bottom flask (100 mL) and refluxed at 80 °C in the presence of ethanol (1.5 mL) for 5-7 h. The resulting crude products were purified and used in the second stage of the reaction. The general reaction mechanism is shown in Figure 1.



R: 3-OCH3, 4-OH, 5-NO2; 4-N(CH3)2; 2,4-Cl2; 2,4-F2; 4-Br

Figure 1. Synthesis of 2-benzylidene malononitrile compounds.

Synthesis of pyridopyrimidine and pyrazolopyridine compounds

Benzylidene malononitrile compounds (1 mmol) and 6-amino-1,3-dimethyl uracil (1 mmol) or 5-amino-1-phenyl-3-methyl-1*H*-pyrazole (1 mmol) compounds in 1.5 mL ethyl alcohol are dissolved (Figure 2).

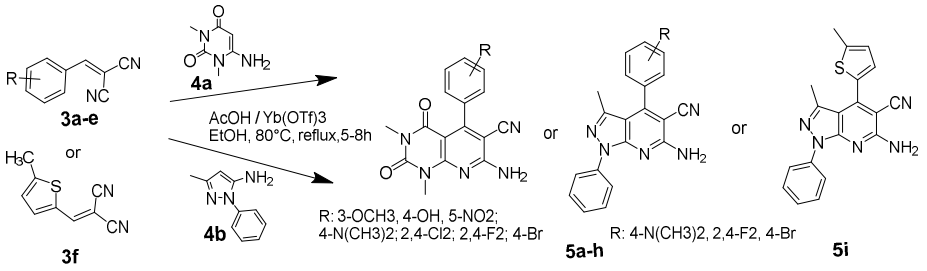
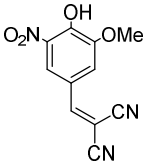
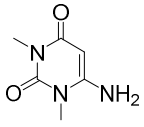
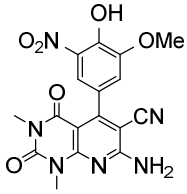
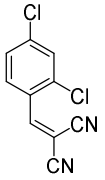
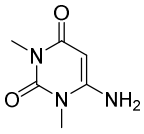
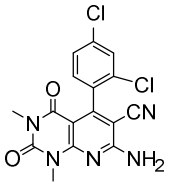
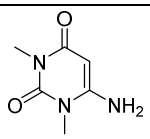
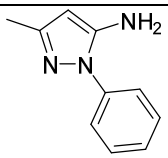
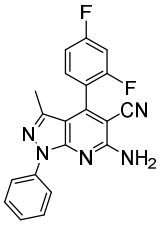
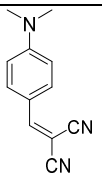
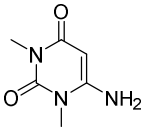
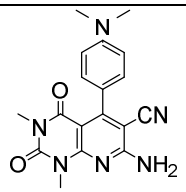
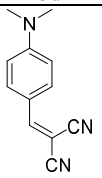
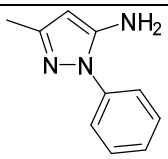
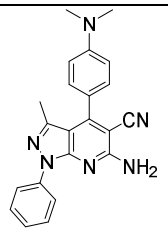
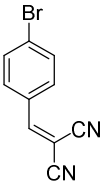
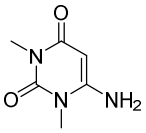
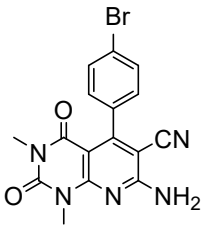
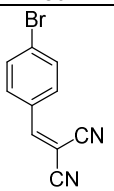
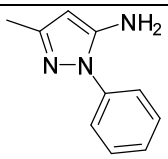
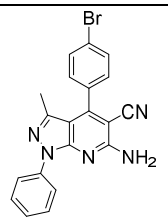


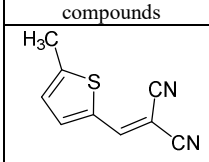
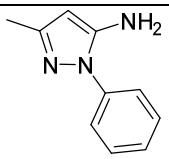
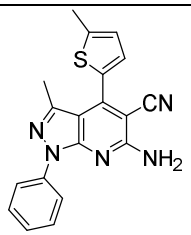
Figure 2. Reaction mechanism of pyrazolo[3,4-*b*]pyridine and pyrido[2,3-*d*] pyrimidine derivatives.

It was refluxed under $Yb(OTf)_3$ catalyst for approximately 7 hours with the addition of acetic acid (0.1 mL) (Table 1). The solid products obtained from TLC controls were purified by chromatographic methods. Their structures were elucidated by spectroscopic methods.

Table 1. Scope of the reactions and optimization of the reaction conditions.

Benzylidene malononitrile compounds	Intermediate products	Reaction time	Final products	Ref.
 **3a**	 **4a**	**8h**	 **5a**	This study
 **3b**	 **4a**	**5h**	 **5b**	[13]
		**7h**		This study

Benzylidene malononitrile compounds	Intermediate products	Reaction time	Final products	Ref.
3c	**4a**		**5c**	
 **3c**	 **4b**	**7h**	 **5d**	[14]
 **3d**	 **4a**	**7h**	 **5e**	[15]
 **3d**	 **4b**	**7h**	 **5f**	[16]
 **3e**	 **4a**	**8h**	 **5g**	[15]
 **3e**	 **4b**	**7h**	 **5h**	[17]

Benzylidene malononitrile compounds	Intermediate products	Reaction time	Final products	Ref.
 **3f**	 **4b**	**8h**	 **5i**	[14]

Elucidation of the chemical structure of compounds

*4-Hydroxy-3-methoxy-5-nitrophenyl)methylidene propanedinitrile (**3a**)*. Bright yellow crystals; m.p. 151.7-153.5 ºC; yield 77.1%; FTIR (ATR) ν/cm^{-1} 3253, 3079, 2233, 1613, 1594, 1570, 1531, 1392, 1462, 1448, 1333, 1384, 1133, 806, 759; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.00 (s, 3H, CH_3), 7.25 (s, 1H), 7.65 (s, 1H), 7.98 (d, 1H, *J* 9.0 Hz, ArH), 8.03 (d, *J* 9.01 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 57.09, 82.76, 112.14, 112.84, 114.59, 114.88, 122.02, 150.46, 151.16, 157.03; MS (ESI) *m/z*, calcd. for $C_{11}H_7O_4N_3$ $[M + H]^+$: 246.05, found: 246.17; anal. calcd. for $C_{11}H_7O_4N_3$: C 53.88, H 2.88, N 17.14, found: C 54.12, H 2.91, N 17.18.

*7-Amino-5-(4-hydroxy-3-methoxy-5-nitrophenyl)-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (**5a**)*. Orange crystals; m.p. 188.9-190.4 ºC; yield 10.3%; FTIR (ATR) ν/cm^{-1} 3396, 3346, 3225, 2183, 1705, 1650, 1540, 1496, 1375, 1302, 1234, 788, 771, 752; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.03 (s, 3H, CH_3), 3.21 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 6.77 (s, 1H, OH), 6.92 (s, 2H, NH_2), 7.35 (s, 1H, Ar), 7.46 (s, 1H, Ar); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 27.51, 29.75, 39.51, 39.72, 39.93, 40.14, 56.75, 75.31, 151.78, 155.32, 161.85; MS (ESI) *m/z*, calcd. for $C_{17}H_{14}O_6N_6$ $[M + H]^+$: 399.11, found: 399.19; anal. calcd. for $C_{17}H_{14}O_6N_6$: C 51.26, H 3.54, N 21.10, found: C 51.35, H 3.57, N 21.15.

*7-Amino-5-(2,4-dichlorophenyl)-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (**5b**)*. White crystals; m.p. 246.7-247.1 ºC; yield 38.7%; FTIR (ATR) ν/cm^{-1} 3332, 3235, 3071, 2973, 2221, 1705, 1644, 1547, 1433, 1227, 882, 769; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.30 (s, 3H, CH_3), 3.65 (s, 3H, CH_3), 7.12 (d, 1H, Ar), 7.36 (s, 2H, NH_2), 7.53 (d, 1H, Ar); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 28.37, 30,23, 58.41, 89.64, 100.64, 114.46, 127.57, 128.91, 129.64, 132.29, 134.75, 151.06, 153.86, 155.85, 160.06; MS (ESI) *m/z*, calcd. for $C_{16}H_{11}Cl_2N_5O_2$ $[M + H]^+$: 376.04, found: 376.20; anal. calcd. for $C_{16}H_{11}Cl_2N_5O_2$: C 51.08, H 2.95, Cl 18.85, N 18.62, found: C 51.11, H 2.97, Cl 18,86, N 18.65.

*7-Amino-5-(2,4-difluorophenyl)-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetra-hydropyrido[2,3-d]pyrimidine-6-carbonitrile (**5c**)*. White crystals; m.p. 128-129 ºC; yield: 9.5%; FTIR (ATR) ν/cm^{-1} 3377, 3274, 2919, 2254, 1655, 1609, 1495, 1371, 1268, 882, 769; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.30 (s, 3H, CH_3), 3.49 (s, 3H, CH_3), 6.85 (m, 1H, Ar-H), 6.91 (m, 1H, Ar-H), 6,94 (m, 1H, Ar-H), 7.27 (s, 2H, NH_2); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 13.34, 20.24, 24.72, 29.88, 36.14, 60.35, 83.78, 104.22, 110.73, 112.85, 149.44, 151.55, 162.20, 171.22; MS (ESI) *m/z*, calcd. for $C_{16}H_{11}F_2O_2N_5$ $[M + H]^+$: 344.10, found: 344.29; anal. calcd. for $C_{16}H_{11}F_2O_2N_5$: C 55.98, H 3.23, F 11.07, N 20.40, found: C 56.06, H 3.28, F 11.14, N 20.45.

6-Amino-4-(2,4-difluorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile ***(5d)***. White crystal; m.p. 148.6-150.5 ºC; yield: 12.5%; FTIR (ATR) ν/cm^{-1} 3351, 3215, 3079, 2224, 1724, 1615, 1596, 1507, 1140, 862, 755; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.03-2.08 (s, 3H, CH_3), 3.31 (d, 2H, *J* 8.2 Hz, ArH), 4.91-5.49 (broad, s, 2H, NH_2), 7.06-7.28 (m, 2H, Ar-H), 7.46 (m, 3H, Ar-H), 8.09 (d, *J* 8.4 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 13.92-14.20, 29.31, 59.68-60.44, 77.34, 121.156, 129.00, 138.76, 143.96-145.11, 150.91, 158.34; MS (ESI) *m/z*, calcd. for $C_{20}H_{13}F_2N_4$ $[M + H]^+$: 362.12, found: 362.36; anal. calcd. for $C_{20}H_{13}F_2N_4$: C 66.48, H 3.63, F 10.52, N 19.38, found: C 66.58, H 3.67, F 10.60, N 19.42.

7-Amino-5-(4-dimethylamino)phenyl-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]-pyrimidine-6-carbonitrile ***(5e)***. Orange crystals; m.p. 180.9-182.5 ºC; yield: 59.8%; FTIR (ATR) ν/cm^{-1} 2920, 2206, 1607, 1559, 1384, 1175, 861, 726; ^{1}H NMR ($CDCl_3$, 500 MHz) δ3.35 (s, 3H, CH_3), 3.60 (s, 3H, CH_3), 5.75 (m, 1H, Ar-H), 6.71 (m, 1H, Ar-H), 7.47 (m, 1H, Ar-H), 7.81 (s, 2H, NH_2); ^{13}C NMR (DMSO-d_6, 125 MHz) δ40.14, 76.73, 77.04, 77.36, 111.60, 114.94, 116.01, 133.83, 154.02, 158.15; MS (ESI) *m/z*, calcd. for $C_{18}H_{18}O_2N_6$ $[M + H]^+$: 351.16, found: 351.37; anal. calcd. for $C_{18}H_{18}O_2N_6$: C 61.70, H 5.18, N 23.99, found: C 61.72, H 5.22, N 24.04.

[6-Amino-4-(4-dimethylamino)phenyl]-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile ***(5f)***. Reddish crystals; m.p. 168-170.5 ºC; yield: 48.6%; FTIR (ATR) ν/cm^{-1} 2917, 2206, 1596, 1508, 1389, 1202, 695, 754; ^{1}H NMR ($CDCl_3$, 500 MHz) δ2.17-2.93 (s, 3H, CH_3), 3.57-5.16 (d, 2H, *J* 8.2 Hz, ArH), 6.70-6.76 (broad, s, 2H, NH_2) 7.17-7.25 (m, 2H, ArH), 7.37 (m, 3H, ArH), 7.48 (d, *J* 8.4 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 11.92-13.65, 22.28, 29.87-34.73, 40.61, 79.63, 101.80, 112.46, 123.59-128.75, 138.08, 142.54, 147.71-149.43; MS (ESI) *m/z*, calcd. for $C_{22}H_{20}N_6$ $[M + H]^+$: 369.18, found: 369.43; anal. calcd. for $C_{22}H_{20}N_6$: C 71.72, H 5.47, N 22.81, found: C 71.74, H 5.50, N 22.76.

7-Amino-1,3-dimethyl-5-(4-bromophenyl)-2,4-dioxo-1,2,3,4-tetra-hydropyrido[2,3-d]pyrimidine-6-carbonitrile ***(5g)***. White crystals; m.p. 96.9-98 °C; yield: 4.6%; FTIR (ATR) ν/cm^{-1} 3308, 3218, 3094, 2916, 2214, 1713, 1660, 1613, 1552, 1373, 1227, 820, 752; ^{1}H NMR ($CDCl_3$, 500 MHz) δ3.31 (s, 3H, CH_3), 3.66 (s, 3H, CH_3), 5.60 (s, broad, 2H, NH_2), 7.15-7.28 (m, 2H, ArH), 7.56-7.80 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 29.93, 36.45, 83.47, 112.14, 122.48, 128.95, 129.06, 130.47, 131.87, 132.19, 132.50, 133.31, 158.44; MS (ESI) *m/z*, calcd. for $C_{16}H_{12}BrO_2N_5$ $[M + H]^+$: 386.02, found: 386.20; anal. calcd. for $C_{16}H_{12}BrO_2N_5$: C 49.76, H 3.13, Br 20.69, N 18.13, found: C 49.78, H 3.15, Br 20.70, N 8.95.

6-Amino-4-(4-bromophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile ***(5h)***. Light yellow crystals; m.p. 151.3-152 ºC; yield: 55.8%; FTIR (ATR) ν/cm^{-1} 3457, 3364, 3000, 2921, 2251, 1620, 1567, 1376, 1027, 820, 752; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.24 (s, 3H, CH_3), 7.23-7.30 (d, 2H, *J* 8.2 Hz, ArH), 7.38 (broad, s, 2H, NH_2) 7.44-7.54 (m, 2H, ArH), 7.59-7.61 (m, 3H, ArH), 8.11 (d, *J* 8.4 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ12.96, 42.20, 100.20, 112.46, 122.52, 124.60, 128.14, 128.90, 129.69, 132.51, 135.09, 137.56, 142.41, 147.42; MS (ESI) *m/z*, calcd. for $C_{20}H_{14}BrN_5$ $[M + H]^+$: 404.05, found: 404.26; anal. calcd. for $C_{20}H_{14}BrN_5$: C 59.42, H 3.49, Br 19.76, N 17.32, found: C 59.44, H 3.50, Br 19.76, N 17.30.

6-Amino-3-methyl-4-(5-methylthiophene-2-yl)-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbo-nitrile ***(5i)***. Orange oily liquid; m.p. 167.5-169 ºC; yield: 9.2%; FTIR (ATR) ν/cm^{-1} 2920, 2206, 1607, 1559, 1384, 1175, 861, 726; ^{1}H NMR ($CDCl_3$, 500 MHz) δ1.34-1.43 (s, 3H, CH_3), 2.48-2.61 (d, 2H, *J* 8.2 Hz, ArH), 6.89 (broad, s, 2H, NH_2) 7.27-7.51 (m, 2H, ArH), 7.59-7.61(m, 3H, ArH), 8.09 (d, *J* 8.4 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ14.99, 15.41, 29.75, 31.55, 76.79, 77.06, 77.38, 121.54, 125.71, 126.09, 126.24, 128.75, 129.00; MS (ESI) *m/z*, calcd. for

$C_{18}H_{14}N_5S$ $[M + H]^+$: 346.11, found: 346.42; anal. calcd. for $C_{18}H_{14}N_5S$: C 66.07, H 4.38, N 20.28, S 9.28, found: C 66.10, H 4.41, N 20.25, S 9.24

Mutagenic and antimutagenic effects of ***5a–5i***

In this study, the mutagenic, and antimutagenic properties of pyrazolo[3,4-*b*]pyridine and pyrido[2,3-*d*]pyrimidine derivatives were investigated in vitro using the Ames/Salmonella assay. Today, this test is also used to investigate the mutagenic/carcinogenic, and antimutagenic/anticarcinogenic effects of potential drugs [18-22]. The genotoxicity results showed that none of the tested compounds were mutagenic up to the highest tested concentration of 1.0 mM per plate (Table 2).

Table 2. Mutagenicity test results for the pyrazolo[3,4-*b*] pyridine and pyrido[2,3-*d*] pyrimidine derivatives (**5a-5i**) in the Ames assay [*TA97a*, *TA98* and *TA100*].

Compd.	Dose level	Number of revertant per plate, and MI (n = 3)					
		TA97a		*TA98*		*TA100*	
	(mM/plate)	Mean±SD[a]	MR	Mean±SD[a]	MR	Mean±SD[a]	MR
5a	0.2	29.4 ± 3.2	0.98	28.7 ± 3.6	0.91	39.1 ± 3.5	1.02
	0.4	30.1 ± 2.9	1.00	29.3 ± 4.2	0.92	37.9 ± 4.1	0.98
	0.6	29.6 ± 2.2	0.98	28.8 ± 5.0	0.91	36.8 ± 2.6	0.96
	0.8	29.0 ± 3.0	0.96	30.6 ± 3.6	0.97	36.2 ± 3.7	0.94
	1.0	30.2 ± 3.4	1.00	31.0 ± 3.5	0.98	36.5 ± 3.5	0.95
5b	0.2	28.2 ± 1.8	0.94	30.6 ± 4.6	0.97	38.6 ± 3.4	1.00
	0.4	28.8 ± 2.6	0.96	31.7 ± 3.9	1.00	40.5 ± 2.9	1.05
	0.6	28.7 ± 3.1	0.95	31.5 ± 4.3	0.99	35.8 ± 3.2	0.93
	0.8	29.2 ± 2.7	0.97	32.2 ± 2.8	1.02	36.0 ± 2.6	0.94
	1.0	28.8 ± 3.0	0.94	33.0 ± 3.2	1.04	39.4 ± 3.6	1.02
5c	0.2	27.4 ± 1.8	0.91	32.7 ± 5.7	1.03	36.7 ± 2.5	0.95
	0.4	29.9 ± 2.7	0.99	32.5 ± 4.9	1.03	38.9 ± 3.1	1.01
	0.6	29.0 ± 3.0	0.96	32.0 ± 4.2	1.01	37.8 ± 3.4	0.98
	0.8	30.3 ± 3.1	1.01	32.3 ± 4.1	1.02	38.6 ± 4.0	1.00
	1.0	29.5 ± 2.9	0.98	32.6 ± 3.8	1.03	39.2 ± 3.8	1.02
5d	0.2	29.8 ± 3.4	0.99	30.4 ± 4.2	0.96	37.6 ± 3.5	0.98
	0.4	30.0 ± 2.8	1.00	31.0 ± 3.9	0.98	39.0 ± 2.9	1.01
	0.6	29.0 ± 1.6	0.96	30.8 ± 3.8	0.97	39.4 ± 2.8	1.02
	0.8	30.8 ± 2.4	1.02	31.6 ± 4.5	1.00	40.1 ± 3.7	1.04
	1.0	30.6 ± 2.4	1.02	33.7 ± 2.9	1.06	39.6 ± 2.6	1.03
5e	0.2	29.2 ± 2.6	0.97	31.5 ± 3.5	0.99	37.5 ± 3.9	0.97
	0.4	30.1 ± 2.7	1.00	32.1 ± 2.8	1.01	37.4 ± 3.7	0.97
	0.6	30.2 ± 2.2	1.00	30.8 ± 3.2	0.97	38.0 ± 2.7	0.99
	0.8	30.6 ± 1.8	1.02	31.6 ± 4.4	1.00	39.5 ± 2.3	1.03
	1.0	30.7 ± 1.4	1.02	32.0 ± 4.0	1.01	39.8 ± 2.6	1.03
5f	0.2	28.7 ± 1.9	1.03	32.2 ± 3.8	1.02	38.3 ± 2.9	0.99
	0.4	29.0 ± 2.0	0.95	31.0 ± 3.6	0.98	38.6 ± 2.8	1.00
	0.6	29.6 ± 2.5	0.98	33.2 ± 2.6	1.05	39.2 ± 3.4	1.02
	0.8	31.1 ± 2.7	1.03	32.3 ± 2.5	1.02	39.1 ± 2.0	1.02
	1.0	30.4 ± 3.2	1.01	30.2 ± 4.8	0.95	40.2 ± 5.4	1.04
5g	0.2	30.5 ± 2.6	1.01	29.6 ± 4.1	0.97	38.5 ± 2.3	1.00
	0.4	29.7 ± 1.7	0.99	30.7 ± 3.9	0.97	39.4 ± 4.6	1.02
	0.6	30.6 ± 3.8	1.02	28.8 ± 4.7	0.98	39.1 ± 2.5	1.02
	0.8	30.8 ± 3.2	1.02	30.5 ± 3.0	0.95	39.8 ± 4.7	0.99
	1.0	31.2 ± 3.5	1.04	30.4 ± 3.7	1.02	38.7 ± 3.6	1.05
5h	0.2	27.0 ± 1.7	0.93	29.9 ± 4.5	1.04	36.4 ± 2.8	1.07

Compd.	Dose level	Number of revertant per plate, and MI (n = 3)					
	0.4	28.7 ± 2.1	0.95	30.5 ± 4.0	0.96	39.5 ± 5.3	0.99
	0.6	27.9 ± 3.3	0.97	30.9 ± 4.3	0.94	38.8 ± 4.5	1.02
	0.8	28.5 ± 2.9	0.95	31.5 ± 2.7	0.96	38.6 ± 3.0	1.02
	1.0	29.2 ± 3.2	0.97	32.4 ± 4.3	0.98	39.0 ± 4.5	1.03
5i	0.2	29.3 ± 2.7	0.97	31.5 ± 3.4	0.92	36.5 ± 3.9	1.03
	0.4	29.6 ± 1.2	0.98	30.9 ± 3.7	0.95	35.8 ± 4.3	0.93
	0.6	29.5 ± 0.9	0.98	32.7 ± 4.2	0.97	36.9 ± 2.7	0.96
	0.8	28.8 ± 3.4	0.96	31.6 ± 3.1	0.97	37.7 ± 4.1	0.98
	1.0	29.8 ± 1.8	0.99	30.9 ± 2.9	0.98	37.8 ± 3.6	0.98
Negative control							
DMSO	100 µL/plate	30.1 ± 3.4	-	31.7 ± 4.0	-	38.5 ± 2.5	-
Positive control							
NaN_3	10 µL/plate	-	-	-	-	670.5 ± 9.4	17.42
9-AA	40 µL/plate	388.8 ± 7.2	12.92	-	-	-	-
NPDA	2.5 µL/plate	-	-	323.0 ± 6.8	10.19	-	-
[a]Mutagenicity results are expressed as mean ± SD from three independent experiments. For multiple comparisons, one-way ANOVA followed by a post hoc Turkey's HSD test was performed. The level of statistical significance was set to $p < 0.05$.							

For the bacterial reverse mutation test, TA97a, TA98, and TA100 of *S. typhimurium* are recommended for routine use [23]. This indicates that all synthesized pyrazolo[3,4-b]pyridine and pyrido[2,3-d]pyrimidine derivatives can be considered genotoxically safe as they do not cause base substitution, and/or frameshift point mutations in the target DNA [24]. On the other hand, 9-AA, NPDA, and NaN_3 model mutagens for *S. typhimurium* TA97a, TA98, and TA100 were used in the antigenotoxicity assays, respectively. The results of the experiments investigating the antigenotoxic potential of the pyrazolo[3,4-b]pyridine and pyrido[2,3-d]pyrimidine compounds against these mutagens are summarized in Table 3.

Table 3. Antimutagenicity of the pyrazolo[3,4-b] pyridine and pyrido[2,3-d] pyrimidine derivatives (**5a-5i**) against *S. typhimurium* TA97a, TA98 and TA100.

Compound	Dose level	Number of revertant per plate, and MI (n = 3)					
	(mM/plate)	TA97a Mean±SD[a]	Inh.[b] (%)	TA98 Mean±SD[a]	Inh.[b] (%)	TA100 Mean±SD[a]	Inh.[b] (%)
5a	0.2	423.8 ± 7.8	3.2	185.6 ± 4.2	1.3	525.4 ± 6.8	3.0
	0.4	420.6 ± 6.9	3.9	182.0 ± 3.9	3.2	521.2 ± 7.1	3.8
	0.6	425.1 ± 8.5	2.9	176.3 ± 5.6	6.2	519.7 ± 6.7	4.1
	0.8	418.7 ± 9.6	4.3	178.6 ± 4.8	5.0	*422.8 ± 8.9	22.0
	1.0	416.5 ± 5.9	4.8	173.3 ± 7.4	7.8	*375.4 ± 7.4	30.7
5b	0.2	429.8 ± 8.6	1.8	176.2 ± 2.4	6.3	517.4 ± 11.7	4.5
	0.4	425.7 ± 9.1	2.7	172.8 ± 2.7	8.1	515.3 ± 10.7	4.9
	0.6	428.0 ± 7.3	2.2	165.9 ± 4.5	11.8	507.5 ± 7.4	6.3
	0.8	419.9 ± 6.4	4.0	161.1 ± 3.7	14.3	519.3 ± 5.4	4.2
	1.0	415.4 ± 6.1	5.1	154.3 ± 6.1	17.9	512.2 ± 9.2	5.5
5c	0.2	422.4 ± 9.2	3.5	184.1 ± 5.2	2.1	519.3 ± 7.7	4.2
	0.4	420.0 ± 7.6	4.0	183.7 ± 4.8	2.3	515.0 ± 9.0	4.9
	0.6	423.5 ± 7.7	3.2	180.7 ± 4.1	3.9	509.8 ± 5.2	5.9
	0.8	407.4 ± 11.4	6.9	178.5 ± 7.9	5.1	506.1 ± 11.8	6.6
	1.0	411.8 ± 10.6	5.9	177.0 ± 6.5	5.9	517.3 ± 11.1	4.5
5d	0.2	404.2 ± 6.2	7.6	178.2 ± 10.3	5.2	519.8 ± 14.2	4.1
	0.4	398.8 ± 5.8	8.9	172.2 ± 9.2	8.4	517.7 ± 3.7	4.4
	0.6	402.5 ± 9.3	8.0	178.3 ± 7.2	5.2	512.7 ± 9.8	5.4

Compound	Dose level (mM/plate)	Number of revertant per plate, and MI (n = 3)					
		TA97a Mean±SD[a]	Inh.[b] (%)	TA98 Mean±SD[a]	Inh.[b] (%)	TA100 Mean±SD[a]	Inh.[b] (%)
	0.8	395.9 ± 8.4	9.5	176.7 ± 11.6	6.0	507.2 ± 4.4	6.4
	1.0	391.5 ± 9.7	10.5	176.1 ± 9.8	6.3	504.3 ± 8.4	6.9
5e	0.2	408.8 ± 8.1	6.6	177.2 ± 7.8	5.7	508.8 ± 10.4	6.1
	0.4	411.4 ± 7.5	6.0	176.0 ± 8.1	6.4	518.2 ± 14.0	4.4
	0.6	400.5 ± 6.7	8.5	175.8 ± 5.9	6.5	511.5 ± 10.8	5.6
	0.8	*342.8 ± 9.4	21.7	172.3 ± 4.5	8.4	503.0 ± 8.3	7.2
	1.0	*303.0 ± 8.9	30.8	170.2 ± 8.6	9.5	507.6 ± 10.4	6.3
5f	0.2	422.8 ± 7.4	3.4	175.2 ± 9.2	6.8	510.2 ± 12.4	5.8
	0.4	420.7 ± 9.8	3.9	171.8 ± 6.8	8.6	509.8 ± 8.6	5.9
	0.6	416.8 ± 11,1	4,8	170.5 ± 8.3	9.3	515.7 ± 13.1	4.8
	0.8	381.2 ± 8.3	12.9	169.7 ± 10.7	9.7	501.5 ± 9.7	7.4
	1.0	*259.5 ± 8.7	40.7	172.1 ± 8.8	8.5	510.0 ± 11.4	5.9
5g	0.2	437.1 ± 5.6	0.1	176.0 ± 9.4	6.4	509.2 ± 12.6	6.0
	0.4	438.4 ± 7.1	-	174.5 ± 9.1	7.2	508.3 ± 9.4	6.2
	0.6	437.6 ± 7.4	-	174.7 ± 4.9	7.2	517.3 ± 12.2	4.5
	0.8	431.5 ± 5.2	1.4	176.5 ± 6.4	6.1	510.2 ± 11.8	5.8
	1.0	429.0 ± 4.7	2.0	182.7 ± 10.2	2.8	508.5 ± 9.2	6.2
5h	0.2	425.5 ± 3.5	2.8	181.4 ± 4.9	3.5	513.7 ± 8.8	5.2
	0.4	422.7 ± 4.2	3.4	179.0 ± 3.6	4.8	503.5 ± 8.1	7.1
	0.6	423.0 ± 3.8	3.3	168.6 ± 5.1	10.3	502.3 ± 11.7	7.3
	0.8	420.7 ± 5.9	3.9	*143.3 ± 7.7	23.8	497.8 ± 11.6	8.1
	1.0	421.4 ± 6.5	3.7	*122.5 ± 6.8	34.8	497.2 ± 12.1	8.2
5i	0.2	439.3 ± 7.2	-	172.7 ± 11.7	8.1	510.2 ± 13.7	5.8
	0.4	438.4 ± 6.9	-	175.0 ± 8.6	6.9	507.0 ± 8.8	6.4
	0.6	437.9 ± 8.1	-	167.3 ± 4.2	11.0	506.6 ± 7.6	6.5
	0.8	438.2 ± 5.2	-	174.0 ± 9.6	7.4	508.7 ± 6.4	6.1
	1.0	437.2 ± 4.8	0.1	177.5 ± 8.9	5.6	504.8 ± 7.9	6.8
Negative control							
DMSO	100 µL/plate	39.8 ± 2.4	-	19.4 ± 2.8	-	39.1 ± 4.3	-
Positive control							
NaN_3	10 µL/plate	-	-	-	-	541.8 ± 10.9	
9-AA	40 µL/plate	437.6 ± 9.5	-	-	-	-	-
NPDA	2.5 µL/plate	-	-	188.0 ± 7.1	-	-	-

[a]Antimutagenicity results are expressed as mean ± SD from three independent experiments. Statistical significance was assessed using one-way ANOVA followed by a Turkey's HSD test ($^*p < 0.05$). [b]Inh.: Inhibition.

According to Table 3, four compounds (**5a**, **5e**, **5f** and **5h**) were found to have significant ($p < 0.05$) antimutagenic activity. Of these, **5h** had moderate inhibitory activity (34.8%) against NPDA-induced mutation in *S. typhimurium* TA98 test strain at 1.0 mM/plate concentration. **5e** showed moderate antimutagenic activity (30.8% and 21.7%) against 9-AA-induced mutagenesis on TA97a strain at 1.0 and 0.8 mM/plate concentrations, respectively. In the case of **5f**, strong (40.7%) inhibition of 9-AA mutagenesis was obtained at 1.0 mM/plate concentration in TA97a test strain. Additionally, **5a** moderately reduced the mutagenic potential of NaN_3 in strain TA100 (30.7%, 1.0 mM/plate; 22.0%, 0.8 mM/plate).

RESULTS AND DISCUSSION

All compounds synthesized in the study were first purified by column chromatography and crystallization, and their structures were elucidated by spectroscopic methods such as FTIR, ^{1}H NMR, ^{13}C NMR and LC-MS. When we look at the FTIR spectra to elucidate the structures of the

compounds obtained in analytical purity, the peaks observed between 3402 and 3225, 3308 and 3274, 3377 and 3218 cm^{-1} give -NH_2 tensions. Peaks between 3229 and 3046 cm^{-1} reveal the aromatic structure. However, the sharp peaks around 2218 cm^{-1} prove the presence of C≡N (nitrile) in the structure. The peaks around 1750 and 1645 cm^{-1} indicate the amide carbonyls introduced into the structure from the uracil ring. In addition, the singlet peaks around 3.03 and 3.78 ppm in the ^{1}H NMR spectra belong to N-CH_3 protons. In addition, peaks belonging to the aromatic protons of the formed pyridine ring are observed as doublets between 7.56-7.80 and 8.15-8.37 ppm. Looking at the ^{1}H NMR spectrum, peaks around 6.92–7.23 ppm confirm the NH_2 part in the structure of the compound formed. ^{13}C NMR spectra shows that all carbons belonging to the structure are located at appropriate values.

The genotoxic and antigenotoxic properties of the synthesized compounds were investigated in vitro using Ames Salmonella/microsome mutagenicity assay in the concentration range of 0.2-1.0 mM/plate [25]. The results revealed that none of the compounds were mutagenic on three different *Salmonella typhimurium* strains up to the highest tested concentration. Moreover, in our study, **5a**, **5e**, **5f** and **5h** showed significant antigenotoxic effects ranging from moderate to strong against mutagen-induced DNA damage at relatively higher doses. Among them, **5f** had the best potential to inhibit the reversible colony number formed by 9-aminoacridine (9-AA) and the maximum inhibition rate was 40.7% for 1.0 mM/plate. In summary, nine pyrazolo[3,4-b]pyridine and pyrido[2,3-d]pyrimidine derivatives (**5a-5i**) did not cause mutagenic effects in *Salmonella* strains; this indicates that these compounds did not cause frameshift (TA97a and TA98) and base pair substitution (TA100). Based on these results, **5a–5i** can be considered genotoxically safe at the tested concentrations (up to 1.0 mM per plate). However, at a dose of 1.0 mM per plate, **5a**, **5e**, **5f** and **5h** showed moderate DNA protective activity against the mutagenic activities of NaN_3 (30.7%), 9AA (30.8%), 9AA (40.7%) and NPDA (34.8%), respectively, while **5f** induced strong antimutagenicity against 9-AA. It can be concluded from this that these compounds, especially **5f**, can be used as antimutagenic agents that may play an important role in cancer prevention. However, further studies are needed to fully elucidate their antimutagenic activity's exact mechanisms of action.

CONCLUSION

Pyrazolo[3,4-b]pyridine derivatives and pyrido[2,3-d]pyrimidine derivatives from the heterocyclic compound class are known to exhibit effects such as antifolate, tyrosine kinase, antimicrobial, calcium channel antagonist, anti-inflammatory, analgesic, antihypertensive, antileishmanial, tuberculostatic, and diuretic. In the first stage of this study, which consists of two main parts, 2-benzylidene malononitrile compounds were synthesized by combining malononitrile with various substituted aldehydes using only ethanol and Knoevenagel condensation in a catalyst-free environment. In this stage, compound **3a** was added to the literature as an original substance. In the second stage, these synthesized compounds were reacted with both 5-amino-3-methyl-1-phenylpyrazole and 6-amino-1,3-dimethyluracil to synthesize pyrido[2,3-d]pyrimidine and pyrazolo[3,4-b]pyridine compounds. In these reactions, ytterbium(III) triflate [$Yb(OTf)_3$] was used as a catalyst for the synthesis of **5a**, **5b**, **5e**, **5f**, **5g**, **5h**, **5i** and acetic acid was used for the synthesis of **5a**, **5b**, **5e**, **5f**, **5g**, **5h**, **5i**. In this stage, compounds **5a** and **5c** were added to the literature as original substances. These preliminary data shared in the results section on pyrazolo[3,4-b]pyridine and pyrido[2,3-d]pyrimidine derivatives can be used as a starting point for chemical modifications to develop more effective antimutagenic agents, thus providing new treatment options for the prevention of mutation-associated diseases such as cancer.

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