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Synthesis of novel ethyl 2,4-disubstituted 8-(trifluoromethyl)pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine-9-carboxylate derivatives as promising anticancer agents

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ABSTRACT

A series of novel pyrido[2′,3′:3,4] pyrazolo[1,5-*a*]pyrimidine derivatives **6**–**9** were prepared in single step starting from 3-amino-6-(trifluoromethyl)-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylate **5** on reaction with symmetrical and unsymmetrical aliphatic and aromatic 1,3-diketones/α,β unsaturated ketones/α,β unsaturated keto ethers under conventional method. All the final compounds **6a**–**c**, **8a**–**b** and **9a**–**l** were screened for anticancer activity against five human cancer cell lines such as PC-3 (CRL-1435), MDA-MB-231 (HTB-26), Hep G2 (HB-8065), HeLa (CCL-2) and normal HUVEC (CRL-1730). Compounds **8a**, **9f** and **9k** which showed promising anticancer activity have been identified. Further, the promising compounds (**8a** and **9f**) were able to inhibit the human topoisomerase I (TopI) activity similar to that of camptothecin.

Cancer is considered as one of the most debilitating disease causes health problem in human beings all over the world.[1,2] Although many classes of drugs were used for the treatment, still there is a challenge to identify safe and effective anticancer compounds as drugs. The high proliferative nature is an important feature of cancer cells. In this regard, topoisomerase I (TopI), whose activity is higher in cancer cells as compared to healthy cells, is one of the most important molecular target for the design of various anticancer drugs.[3,4] Several heterocyclic compounds were intensively studied to discover new anticancer agents.[5–7] Pyrazolo[3,4-*b*]pyridine frame work is identified as one of the most important classes of heterocyclic compounds because of their significant and versatile biological and pharmacological properties.[8–10] In the last decade, some heterocycles of this class have exhibited antiviral,[11,12] antileishmanial,[13] GSK-3 inhibitor[14] and antimicrobial properties.[15]

Pyrazolo[3,4-*b*]pyridine nucleus[16] also present in diaryl pyrazolopyridine,[17] isoxazolopyrazolopyridine[18] displayed anxiolytic and hypotensive activities. In addition, pyrazolo[3,4-*b*]pyridine derivatives with a trifluoromethyl group were known to possess multidrug action with antimalarial activity.[19] Recently, it was observed that the presence of fluorine[20] or trifluoromethyl[21,22] group at a strategic position in heterocycles can modulate the physical, chemical and biological properties. Further, it is also documented that the trifluoromethyl group in a molecule can influence the increased lipophilicity, greater cell permeability and resistance to enzyme degradation.[23]

Based on the broad range of pharmacological activity of this class of compounds and in continuation of our efforts,[24–26] prompted us to synthesize the novel trifluoromethyl substituted pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine derivatives and to evaluate their anticancer activity against five human cancer cell lines. The compounds which showed promising activity have been identified.

Ethyl 5-cyano-6-oxo-2-(trifluoromethyl)-1,6-dihydropyridine-3-carboxylate **3**[30] was prepared starting from β-ketoester, on reaction with triethylorthoformate in acetic anhydride at 120 °C followed by reaction with cyanoacetamide in presence of sodium ethoxide in ethanol under reflux conditions. Compound **3** was selectively *O*-alkylated with α-bromoethylacetate in acetone using potassium carbonate as a base under reflux conditions and then reacted with hydrazine hydrate to obtain ethyl 3-amino-6-(trifluoromethyl)-1*H*-pyrazolo[3,4-*b*] pyridine-5-carboxylate **5**. The pyrazolo[3,4-*b*]pyridine **5** is a key intermediate in the synthesis of the final pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine derivatives. Thus,

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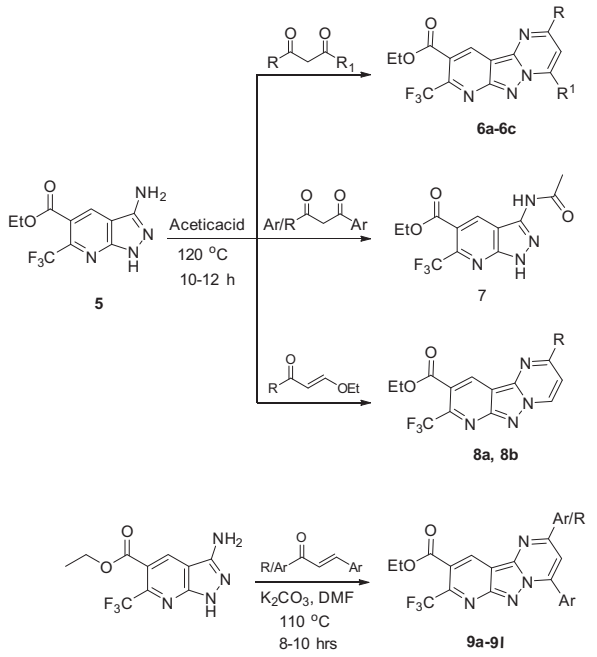
the compound **5** was reacted with symmetrical, unsymmetrical 1,3-diketones (aliphatic and aromatic), α,β-unsaturated ketones and α,β-unsaturated keto ethyl ethers under different set of conditions afforded the final pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine derivatives **6a–c**, **8a–b** and **9a–l**. Initially, compound **5** on reaction with aliphatic symmetrical 1,3-diketones in presence of acetic acid at 120 °C, reaction proceeded smoothly and the anticipated pyrido [2′,3′:3,4]pyrazolo[1,5-*a*] pyrimidine derivatives, **6a** and **6c**, were obtained. While compound **5** on reaction with trifluoroacetyl acetone (unsymmetrical 1,3-diketone) resulted exclusively in the formation of compound **6b.** It is attributed to the enolization of carbonyl carbon attached to CF_3 function as a result other carbonyl attached to CH_3 is available for initial nucleophilic attack thereby exclusive formation of compound **6b** is observed. This corroborates with the observations made in the earlier reports.[27,28] However, in case of aromatic 1,3-diketones, an unexpected acylated product **7** was formed which was confirmed by NMR and mass spectra. This may be attributed to the low reactivity of aromatic 1,3-diketones and solvent only reacted to give product **7**. In order to get the desired product, the reaction of compound **5** was repeated with aromatic 1,3-diketone under different set of conditions, showed no reaction.

Alternately, compound **5** was treated with aromatic 1,3-diketone in absence of acetic acid in DMF solvent at 110 °C and potassium carbonate as base also could not give the expected product except the recovery of the starting material. To synthesize the aryl substituted pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine derivatives **9a–l**, we adopted a different strategy that, the aromatic α,β-unsaturated ketones on treating with compound **5** in presence of potassium carbonate in DMF at 110 °C resulted aromatic substituted pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine derivatives **9a–l**. The synthetic sequence involved in the synthesis of target compounds is outlined in Scheme 1 and 2 and the product formed are presented in Table 1.

The compounds **6a–c**, **8a–b** and **9a–l** were screened for cytotoxicity[31] against five human cancer cell lines, namely, PC3: Human prostate cancer (CRL-1435), MDA-MB-231: human breast adenocarcinoma cancer (HTB-26), HepG2: human liver cancer (HB-8065), HeLa: human cervical cancer (CCL-2), and HUVEC: normal human umbilical vein endothelial cells (CRL-1730) using 5-fluorouracil (5-FU) as positive and DMSO as negative controls, respectively. All the test compounds showed above 60% inhibition and subsequently, their IC_{50} values were calculated from the dose–response curves (Table 2). From the data reported in Table 2, most of the prepared compounds exhibited significant anticancer activity on all the tested cell lines.

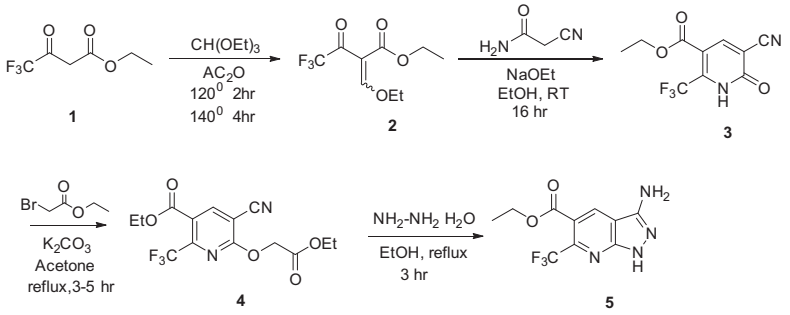
Compounds **6a**, **8a**, **9e–g** and **9j–l** showed promising activity against all the five human cancer cell lines. Compounds **6b** and **9a** showed moderate cytotoxicity against HeLa and MDA-MB-231 cancer cell lines. Among all the compounds, compound **8a**, **9f** and **9k** exhibited very good cytotoxicity against all the tested



Scheme 2. Synthesis of pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine derivatives **6a–c, 8a–b, 9a–l.**

cancer cell lines with concentrations ranging between 10.3 and 17.2 μM. Compound **8a** showed significant activity against PC3, HeLa and HepG2 cell lines, whereas the compound **9f** showed good cytotoxicity against MDA-MB-231 cell line.

The structure–activity relationship (SAR) studies revealed that the compound **8a** which has CH_3 group on pyrimidine ring showed promising cytotoxicity; however, in presence of two methyl groups on the pyrimidine ring showed moderate cytotoxicity in case of compound **6a**. Similarly, when one methyl group is replaced with CF_3 group, the cytotoxicity was further lower in case of compound **6b**. Compound **6c** which has two ethyl groups on pyrimidine ring did not show any cytotoxicity against all the five tested human cancer cell lines upto the maximum tested concentration of 100 μM. Thus, it is concluded that the presence of electron releasing groups with increase in the alkyl chain length on the pyrimidine ring showed no cytotoxicity. Among the compounds (**9a–l**) having aryl substituent on pyrimidine ring, it was observed that the compounds having electron donating groups on the aromatic ring (**9c**, **9d**, **9h** and **9i**) showed no cytotoxicity, whereas electron withdrawing groups on the compounds (**9e–g**, **9j–l**) having aryl substituent on the pyrimidine ring showed very good cytotoxicity; in particular, the compound having OCF_3 group showed promising cytotoxicity. In general, compounds having thiophene and pyrrole



Scheme 1. Synthesis of 3-amino-6-(trifluoromethyl)-1*H*-pyrazolo[3,4-*b*]pyridine-5 carboxylate **5.**

Table 1
Synthesis of pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine derivatives **6a–c, 8a–8b, 9a–l**

S. No.	Compd	R	R^1	Yield (%)	mp (°C)
1	**6a**	CH_3	CH_3	85	158–160
2	**6b**	CH_3	CF_3	87	150–153
3	**6c**	C_2H_5	C_2H_5	88	169–172
4	**8a**	CH_3	—	72	134–136
5	**8b**	CF_3	—	68	168–171
6	**9a**	CH_3	C_6H_5	76	216–217
7	**9b**	C_6H_5	C_6H_5	73	188–190
8	**9c**	C_6H_5	4-OCH_3 C_6H_5	67	153–155
9	**9d**	C_6H_5	4-Cl C_6H_5	69	201–203
10	**9e**	C_6H_5	4-NO_2 C_6H_5	86	215–217
11	**9f**	C_6H_5	C_4H_4N	77	228–230
12	**9g**	4-OCH_3 C_6H_5	4-F C_6H_5	74	140–142
13	**9h**	4-OCH_3 C_6H_5	4-OCH_3 C_6H_5	76	141–143
14	**9i**	-C_4H_3S	2-CH_3 C_6H_5	82	213–215
15	**9j**	-C_4H_3S	4-F C_6H_5	83	219–221
16	**9k**	-C_4H_3S	4-OCF_3 C_6H_5	88	217–219
17	**9l**	-C_4H_3S	4-OCH_3 2,6-di F-C_6H_2	86	239–241

Table 2
In vitro cytotoxicity evaluation of pyrido[2′,3′:3,4]pyrazolo[1,5-*a*]pyrimidine derivatives

Test compound	IC_{50} values (μM)								
	PC3[a]	SI[f]	HeLa[b]	SI[f]	MDA-MB231[c]	SI[f]	Hep G2[d]	SI[f]	HUVEC[e]
6a	22.1 ± 0.36	4.48	18.2 ± 0.28	5.45	20.1 ± 0.42	4.93	21.2 ± 0.32	4.67	99.2 ± 0.24
6b	—	—	25.6 ± 0.32	3.44	24.3 ± 0.26	3.62	—	—	88.2 ± 0.26
6c	—	—	—	—	—	—	—	—	—
8a	11.4 ± 0.12	8.17	10.6 ± 0.15	8.79	12.1 ± 0.18	7.70	11.8 ± 0.24	7.89	93.2 ± 0.14
8b	—	—	—	—	—	—	—	—	——
9a	—	—	29.8 ± 0.36	3.05	28.6 ± 0.28	3.18	—	—	91.1 ± 0.18
9b	—	—	—	—	—	—	—	—	—
9c	—	—	——	—	—	—	—	—	—
9d	—	—	—	—	—	—	—	—	—
9e	16.3 ± 0.24	5.57	18.2 ± 0.18	4.98	12.2 ± 0.19	7.44	14.2 ± 0.24	6.39	90.8 ± 0.25
9f	12.1 ± 0.26	7.86	13.1 ± 0.22	7.26	10.3 ± 0.14	9.24	14.8 ± 0.32	6.43	95.2 ± 0.26
9g	15.9 ± 0.12	5.43	15.8 ± 0.16	5.46	17.2 ± 0.21	5.02	16.1 ± 0.12	5.36	86.4 ± 0.34
9h	—	—	—	—	—	—	—	—	—
9i	—	—	—	—	——	—	—	—	—
9j	15.1 ± 0.18	5.68	15.3 ± 0.18	5.61	17.1 ± 0.14	5.02	16.8 ± 0.12	5.11	85.9 ± 0.32
9k	11.9 ± 0.24	7.55	16.1 ± 0.42	5.58	17.2 ± 0.16	5.22	12.6 ± 0.24	7.13	89.9 ± 0.26
9l	22.8 ± 0.12	4.07	20.4 ± 0.24	4.54	21.1 ± 0.28	4.39	23.5 ± 0.42	3.94	92.8 ± 0.42
5-Fluorouracil (Control)	1.8 ± 0.11	44.22	1.6 ± 0.12	49.75	1.9 ± 0.09	41.89	1.8 ± 0.11	44.22	79.6 ± 0.24

[a] PC3: Human prostate cancer (ATCC No. CRL-1435).
[b] HeLa: Human cervical cancer (ATCC No. CCL-2).
[c] MDA-MB-231: Human breast adenocarcinoma cancer (ATCC No. HTB-26).
[d] HepG2: Human Liver cancer (ATCC No. HB-8065).
[e] HUVEC: Human umbilical vein endothelial cells (ATCC No. CRL-1730).
[f] Selectivity index (SI) = IC_{50} of pure compound in a normal cell line/IC_{50} of the same pure com -pound in cancer cell line, where IC_{50} is the concentration required to inhibit 50% of the cell population.

substituent on pyrimidine ring also exhibited good cytotoxicity. Pyrrole derivative (**9f**) also showed promising cytotoxicity as compared to thiophene derivatives. From the cytotoxicity results, it was observed that the compounds **8a**, **9f** and **9k** exhibited promising cytotoxicity among all the tested compounds. All the compounds when tested on HUVEC (normal cell line), showed IC_{50} values of >80 μM and were thus considered as less or non-cytotoxic. Further, the selectivity index (SI) values were also determined and the values to this regard are represented in Table 2.

Based on the cytotoxicity results of the synthesized compounds, we examined two promising compounds (**8a** and **9f**) for in vitro human DNA Topoisomerase I inhibitory activity using camptothecin (CPT) as positive control (Fig. 1). Based on the results presented in Figure 1, it was observed that the supercoiled (sc) DNA (lane S) was fully relaxed by the enzyme (lane R). Relaxation of sc plasmid substrate, pHOT1, was inhibited upon incubation with the test compounds at two different concentrations (10 and 20 μM) similar to that of camptothecin. The results clearly suggest

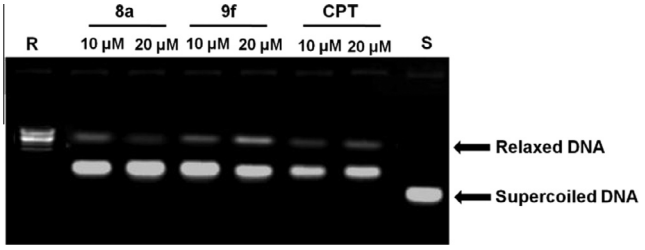


Figure 1. Effect of test compounds (**8a** and **9f**) on human topoisomerase I activity. The supercoiled DNA (pHOT1) substrate was incubated with topoisomerase I; and test compounds (**8a** and **9f**) and camptothecin (CPT) at a concentration of 10 and 20 μM. Relaxation of the DNA was evaluated by 1% ag- -arose gel electrophoresis in the presence of ethidium bromide. R, relaxed DNA and S, supercoiled pHOT1.

that the synthesized compounds (**8a** and **9f**) successfully inhibited the enzymatic activity of topoisomerase I.[29] The human solid tumors generally shows high levels of intracellular accumulation

of topoisomerase I as compared to that of normal tissues, which signifies that controlling the topoisomerase I level is essential for treating cancers.

In conclusion, substituted pyrido[2′,3′:3,4]pyrazolo[1,5-*a*] pyrimidine derivatives were prepared from the key intermediate pyrazolo[3,4-*b*]pyridine **5** and all the final compounds were screened for cytotoxicity against different cancer cell lines. Among all the tested compounds, the compounds **8a**, **9f** and **9k** showed promising cytotoxicity. Further, the promising compounds (**8a** and **9f**) were able to inhibit the Topoisomerase I (TopI) activity similar to that of camptothecin and would thus be an effective target in cancer therapy. Based on the results of the present study, further modification on the pyrazolo [3,4-*b*] pyridine ring may yield a prospective anticancer agent.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.09.062.

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