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ORIGINAL ARTICLE

A new synthetic approach for pyrazolo[1,5-*a*]pyrazine-4(5*H*)-one derivatives and their antiproliferative effects on lung adenocarcinoma cell line

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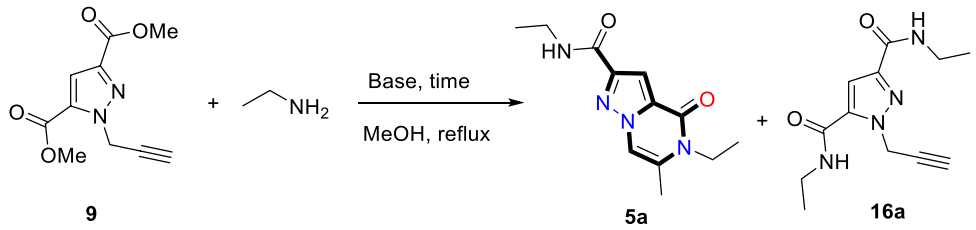
Abstract

Starting from the 3,5-dimethyl pyrazole ring and acetophenone derivatives, five different N-propargylated C-3 substituted pyrazoles were obtained. These derivatives were reacted with different amine derivatives using Cs_2CO_3 in methanol and 11 different pyrazolo [1,5-a] pyrazine-4(5*H*)-one derivatives were obtained, which are not found in the literature. The cytotoxic effects of these derivatives in the A549 cell line were investigated. The 160 µM concentration of two derivatives was found to increase cell death rate to 50%, and two derivatives increased cell death rate by up to 40%. The structure–activity relationship (SAR) study revealed an amide group with a long alkyl chain and benzene ring with a *p*-CF_3 group could be important for efficiency. With theoretical ADMET studies of pyrazolopyrazine derivatives, pharmacokinetic phases were predicted to be suitable.

Graphic abstract



Keywords Alkyne cyclization · Allene · Pyrazole · Lung adenocarcinoma · KRAS mutant lung cancer · ADMET

Introduction

Pyrazole derivatives are appreciated to manifest a wide range of biological qualities such as cannabinoid hCB1 and hCB2 receptor, anti-inflammatory, inhibitors of p38 Kinase, CB1 receptor antagonists, antimicrobial activity [1–4]. Incorporating the heterocyclic ring into prospective pharmaceutical candidates is a significant strategy to obtain activity and safety benefits. As a consequence, much attention has been paid to the design and synthesis of pyrazolopyrazinones, which reveal the HIV-1 integrase inhibitory effect (Fig. 1, compound **2**) [5] and dipeptidyl peptidase-IV inhibitory effect (Fig. 1, compound **1**) [6]. Besides, Miao and his co-workers have announced that pyrazolopyrazinone derivatives showed a vital effect with 22.3 uM on the A549

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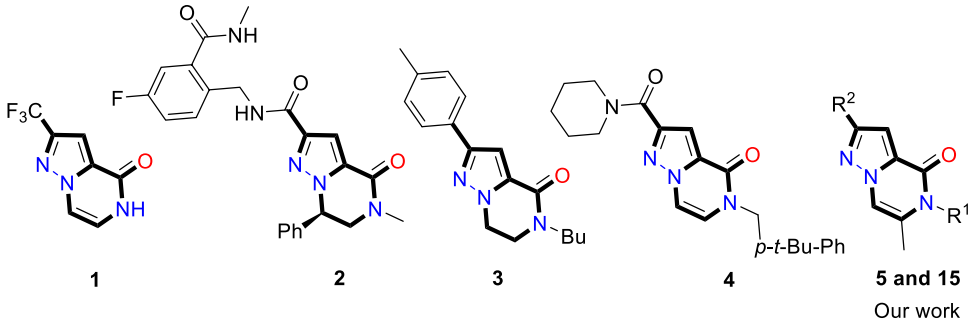
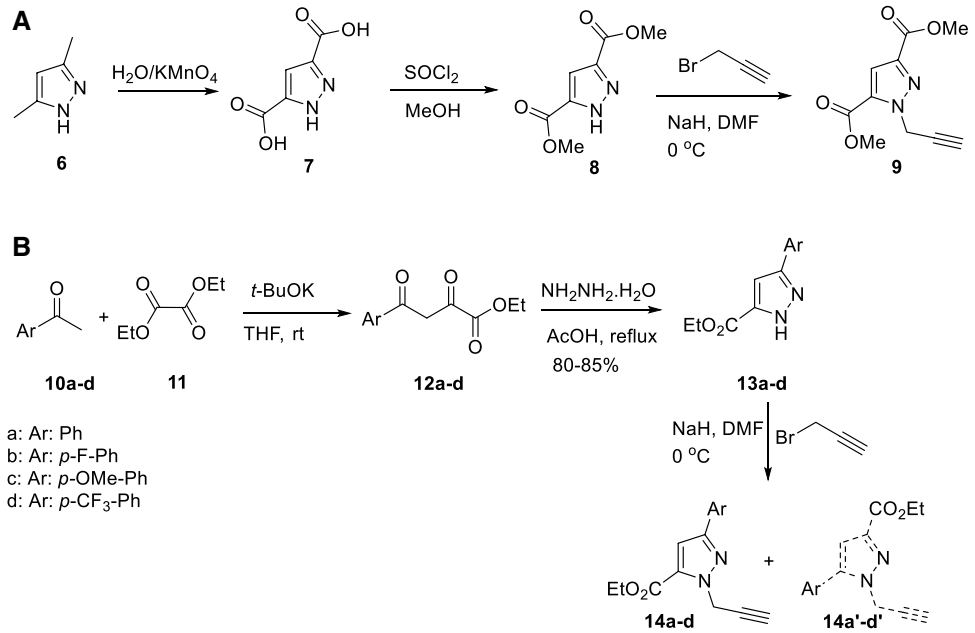


Fig. 1 Representation of some important C-3-substituted pyrazolopyrazinones and our designed molecule

cell line [7]. Surase and co-workers have emerged novel inhibitors of *mycobacterium* ATP synthase in which the central skeleton of found novel inhibitors is pyrazolo [1,5-a] pyrazin-4(5*H*)-one (Fig. 1, compound **4**) [12]. They have pointed out that the skeleton was revealed after the screening of chemically diverse compounds (10.000 compounds) from the compound library. Their synthetic strategy includes alkylation and azidation of diethyl pyrazole-3,5-dicarboxylate followed the reduction of azide functional group using triphenylphosphine. These reagents might give some disadvantages due to their toxicities [13] and the poisoning of catalysts in need [14].

Lung cancer is the most common cancer type all over the World. Lung cancer is classified into three main groups: non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), and carcinoids. NSCLC is further partitioned into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. Among them, adenocarcinoma accounts for 40% of all NSCLC patients [15]. For lung adenocarcinoma, a more appropriate treatment process is started with a personalized treatment by choosing a treatment according to what type of cancer driver mutation is present in the patient's tumor tissue. One of the most common mutations in adenocarcinoma patients, with a 25% rate, is Kirsten rat sarcoma viral oncogene homologous (KRAS) gene mutation [16]. Activated when guaccine triphosphate (GTP) is bound, KRAS stimulates many oncogenic signal transduction pathways in the cell, such as proliferation and survival. KRAS mutations, usually seen in codons 12, 13, and 61, change the protein's conformation and cause it to remain permanently GTP-bound. These mutations are also associated with smoking [17]. One of the conditions that make treatment difficult in patients with KRAS mutant NSCLC is biological heterogeneity observed due to co-mutations. Besides the inadequate chemotherapy response, there is no direct or indirect KRAS inhibitor approved by the FDA. For this reason, the development of inhibitors is of great importance for KRAS mutant lung adenocarcinoma.

Although there are some reported studies about the pyrazolopyrazinone framework, it is barely known. Therefore,



Scheme 1 Synthesis of starting materials. **a** compound **9**; **b** compounds **14a–d**

we have focused on C-3 substituted pyrazolopyrazinone due to their pharmaceutical influence (Fig. 1, compounds **5** and **15**).

In this study, we would like to introduce the synthesis and structural characterization of novel C-3 substituted pyrazolo[1,5-*a*]pyrazin-4(5*H*)-ones and their antiproliferative effects on A549, which is KRAS mutant lung adenocarcinoma cell line.

Result and discussion

Chemistry

The synthesis of starting materials for pyrazolo[1,5-*a*] pyrazin-4(5*H*)-one derivatives was fulfilled as displayed in Scheme 1.

We have followed two different reaction pathways to get starting materials (**9** and **14a–d**). In the first way, drawn in Scheme 1A, the commercially available 3,5-dimethyl pyrazole (**6**) was oxidized with $KMnO_4$ to obtain the pyrazole-carboxylic acid **7** derivative. The second stage was a Schotten–Baumann type reaction using methanol and thionyl chloride, where the 3,5-dicarboxylate-pyrazole derivative **8** was formed.

In the second reaction we carried out to obtain the other C-3 substituted pyrazole ring, the ketoester derivative (**12a–d**) was obtained by the reaction of acetophenone derivatives (**10a–d**) with diethyl oxalate (**11**) in basic medium. Then the cyclization reaction of this ketoester with hydrazine hydrate was used (Scheme 1B). The final step of both reactions to reach our starting materials was the nitrogen atom's propargylation of the pyrazole ring under the basic medium.

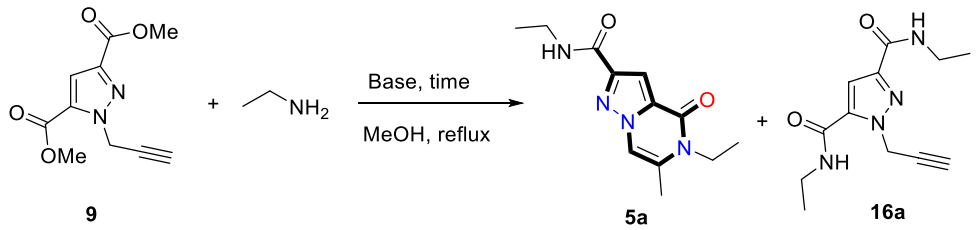
We have studied optimum reaction condition using compound **9**, and it was reacted with ethylamine under different reaction conditions (Table 1).

We did some experiments to achieve optimum conditions and found that the reaction was going too slowly to obtain the cyclic product without base (Entry 3, Table 1). The use of excess amines had a high effect on the cyclic product yield, resulting from the initiation of allene isomerization. The use of an inorganic base has been critical for cyclization (Entries 4–5, Table 1). When Na_2CO_3 was used, it was observed that the cyclization product was 75%, and the amide derivative remained in the medium as 25%. This can be concluded that Na_2CO_3 does not quantitatively convert the propargyl group in the amide molecule to its allen isomer (crucial for cyclization) in the given time. It was, therefore, remarkable to say that Cs_2CO_3 is the right choice.

With optimum conditions in hand, we want to extend the scope of cyclization reaction. Primary amines which bear long alkyl chain, steric-hindered unit, aromatic ring, and allyl unit were utilized. We have heeded that the cyclization protocol can tolerate the long alkyl chain up to heptyl amine and allyl unit resulting in different pyrazolopyrazinone derivatives **5a–f** (Scheme 2A). On the other hand, the starting compound containing benzene ring in the pyrazole ring's C-3 position was reacted with allyl and butylamine to obtain five different pyrazolopyrazinone derivatives **15a–e** in good to high yields (Scheme 2B).

We could not obtain any product other than the starting material and the allene isomer when *t*-butyl amine and aniline were used as an amine source. Although the reaction

Table 1 Preliminary tests for the finding of optimum reaction conditions

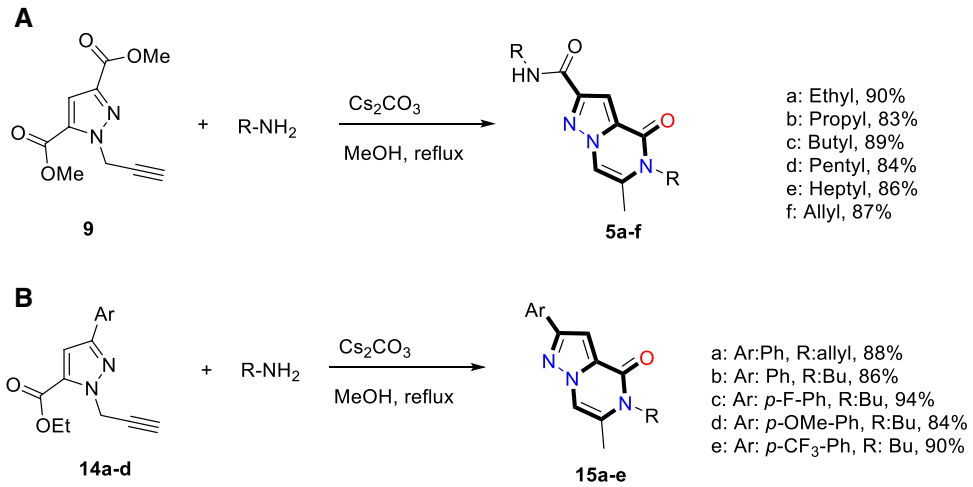


9 **5a** **16a**

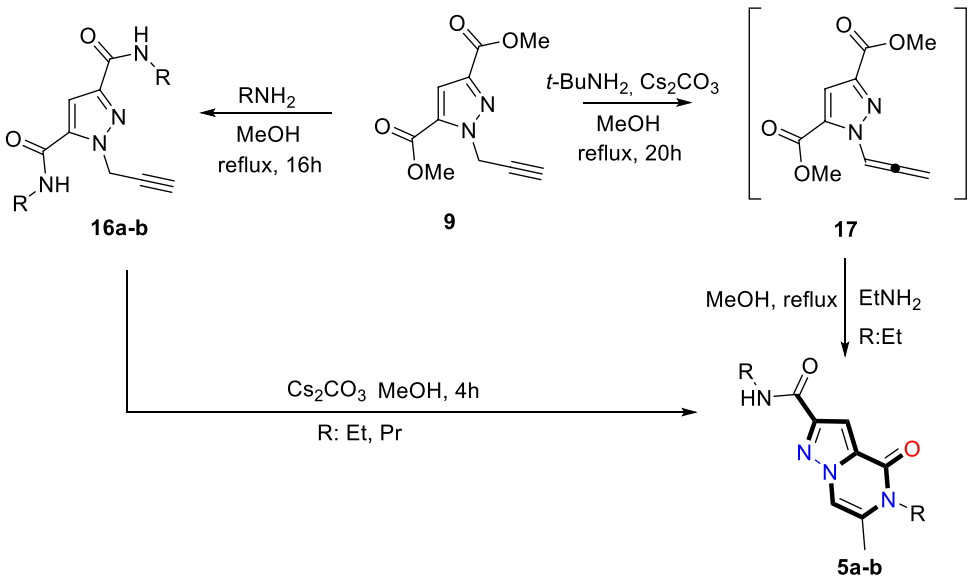
Entry	Equiv. of amine	Base (1 equiv.)	Time (h)	Yield	
				5a (%)	**16a** (%)
1	1	–	6	–	99
2	5	–	6	–	99
3	10	–	24	30	70
4	5	Cs_2CO_3	6	99	–
5	5	Na_2CO_3	6	75	25

was refluxed for 24 h, we received no further reactions. When we evaluated the reaction medium with the ^{1}H-NMR spectrum, we witnessed the allene isomer of the *N*-propargyl-substituted pyrazole (compound **17**).

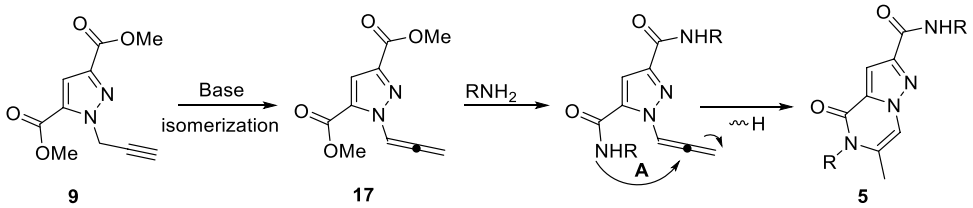
We have noticed that *t*-butyl amine cannot form amide functional groups due to its steric hindrance but only causes allene isomerization (compound **17**). We added 5 equivalents of $EtNH_2$ to the reaction medium without isolating the allene isomer formed in the medium. The reaction medium was refluxed for a further 6 h, and we isolated compound **5a** as the sole product (Scheme 3). Another control experiment included the reaction of compound **16a–b** and Cs_2CO_3. We know that the primary amine leads to amide functionality but did not provide allene isomerization quickly. When the



Scheme 2 Derivatization of pyrazolopyrazinones. **a** compounds **5a–f**; **b** compounds **15a–e**



Scheme 3 Allene isomerization and failure attempt for cyclization



Scheme 4 Proposed reaction mechanism

isolated N-propargyl amide molecules (**16a–b**) reacted with the base, the reaction gave cyclic products quickly (**5a–b**).

Allene isomerization reaction provided a clue for the assumption of reaction mechanism (Scheme 4). We have concluded that the propargyl-allene isomerization was prompted by the base, which has already been reported by us [8, 9]. Propargyl-allene isomerization followed amidation of compound **9** in which nitrogen atom of amide group can attack to middle carbon of allene unit [10, 11]. Attack coming from nitrogen atom resulted in cyclization intermediate, which was finalized with proton transfer. We have remarked that amine with an alkyl chain can afford to make an amide functional group. However, their basicity is not as good as to make an isomerization in a short time. In this manner, Cs_2CO_3 is a valuable base to accomplish isomerization in a short time.

Evaluation of biological activity

Cytotoxicity assay

Some pyrazolopyrazinones were active molecules in the KRAS mutant lung adenocarcinoma cell line, A549. Therefore, the antiproliferative effect of eleven different pyrazolopyrazinone derivatives synthesized in this study was investigated. The antiproliferative effects of molecules on the A549 cell line were examined using the MTT method at 24 and 48 h, and the results are given in Table 2.

In this study, the antiproliferative effect of 11 different molecules with pyrazolopyrazinone core (**5a–f** and **15a–e**) was done (Table 2). Among the molecules, **5c** and **5e**, which cause 50% death of cells, and **5f** and **15e**, which cause 61–62% cell survival, have been identified. Considering the activities of these molecules, valuable information has been obtained dealing with functional groups that should use. The presence of long-chain alkyl group on amide group (**5e**), presence of allyl group instead of propyl group (**5f**) has emerged as important structural groups in derivatives containing amide group (**5a–f**). On the other hand, almost no activity was observed in derivatives containing aromatic rings at the C-3 position of the pyrazolopyrazinone nucleus, except for derivatives containing fluorine atoms (**15c** and **15e**). Compound **15c** has a fluorine atom on the para position of the benzene ring. This partially reduced cell viability by up to 89%. The presence of OMe in the same position did not cause any effects. Replacing the fluorine atom with the CF_3 group (**15e**) decreased the cell viability by up to 62.8%. This suggests that fluorine atoms can make halogen bonds and/or hydrogen bonds at the relevant position. Thanks to these derivatives, it can be said that the effects of some functional groups on the A549 cell line were revealed at the initial level by keeping the pyrazolopyrazinone skeleton fixed.

Table 2 Antiproliferative effect of synthesized molecules on A549 at 24 h

Molecules	Cell viability (%)
5a	76 ± 10.5
5b	75 ± 8.4
5c	51 ± 5.5
5d	57 ± 4.3
5e	51 ± 11.6
5f	61 ± 5.3
15a	97.4 ± 7.8
15b	124 ± 6.3
15c	89 ± 17.9
15d	99 ± 3.1
15e	62.8 ± 12.2
Control (DMSO)	100

Concentration of molecules were 160 μM in DMSO

The importance of bicyclic pyrazole derivatives was also noted in a recently published study [21]. Nasr et al. designed and synthesized pyrazolo[3,4-d] [1–3] triazine derivatives and evaluated for their anticancer activity in Huh-7, Panc-1 and CCRF cells. They showed significant changes in Caspase 3/activity with some compounds in Huh-7 cells. According to the microarray analysis, one compound, 6c, affected apoptosis, metabolism, cell cycle, and tumor growth-related gene expressions in Huh-7 cells.

Theoretical pharmacokinetic study for synthesized molecules

We also wanted to calculate the theoretical ADMET knowledge of the molecule skeleton whose antiproliferative effect was determined. For this purpose, some important ADMET parameters were calculated with the pkCSM program (Table 3) [18]. For this purpose, Caco-2 permeability and small intestinal absorption (Intest. Ab., Table 3), which can be used in the absorption parameter, were calculated. It has been observed that all synthesized molecules have proper Caco-2 permeability. Small intestinal absorption was also calculated to be high, between 86 and 97% (Table 3). The VDss values of the synthesized molecules are not very high. According to this theoretical calculation, it can be stated that the molecules obtained in this study will be found in plasma rather than tissues. The Fu value decreases with the alkyl group's extension on the nitrogen atom, which indicates that the molecule's binding rates to plasma proteins increase. The pyrazolopyrazinone derivative with the heptyl chain (compound **5e**) has the lowest Fu value (Fu:0.08; Table 3). Total clearance of synthesized molecules is varied between 0.40 and 1.60 log(ml/min). Our synthesized molecules are not OCT2 substrate means that they have no potential for

Table 3 Some of ADMET parameters for synthesized molecules (For explanation of abbreviation in Table, please see supporting information)

Entry	Log P	Caco2	Intest. Ab.%	VDss	Fu	CYPs	Total C.	OCT2	hERG I-II	Skin sensitive
5a	0.57	1.40	89	−0.35	0.51	CYP1A2	0.89	No	No	No
5b	1.35	1.36	97	−0.26	0.43	CYP1A2	0.77	No	No	No
5c	1.83	1.32	96	−0.11	0.35	CYP1A2	1.46	No	No	No
5d	2.91	1.35	93	0.058	0.25	CYP3A4, CYP1A2, CYP2C19	1.50	No	No	No
5e	4.48	1.32	92	0.41	0.08	CYP3A4, CYP2C19, CYP2C9	1.60	No	No	No
5f	0.91	1.38	86	−0.28	0.45	CYP1A2	0.8	No	No	No
15a	2.66	1.67	99.8	0.11	0.28	CYP1A2 CYP3A4 CYP2C19	0.61	No	No	No
15b	3.27	1.67	98.7	0.24	0.24	CYP3A4 CYP1A2 CYP2C19	0.62	No	No	No
15c	3.41	1.46	98.2	0.28	0.28	CYP3A4 CYP1A2 CYP2C19 CYP2C9	0.85	No	No	No
15d	3.28	1.37	99.7	0.36	0.25	CYP3A4 CYP1A2 CYP2C19 CYP2C9	0.64	No	No	No
15e	4.29	1.59	96.2	0.27	0.20	CYP3A4 CYP1A2 CYP2C19 CYP2C9	0.40	No	No	No

disposition and clearance effect. One of the most critical parameters for drugs is to be no inhibition on HERGI/II. Calculations show that molecules **5a–f** and **15a–e** do not inhibit hERGI/II and molecules do not occur allergic contact when encountered skin. Theoretical ADMET information was calculated, and the pharmacokinetic information obtained shows us that the molecules synthesized are likely to have suitable pharmacokinetic parameters.

Considering the ADMET and biological activity studies, derivatives that significantly reduce cell viability were **5c, 5e, 5f** and **15e**. Some comments can be made considering the important ADMET parameters of these derivatives given in Table 3.

1. None of these derivatives are hERG inhibitors as cared for in a candidate drug molecule.
2. Log P values were found to be between a suitable range when Lipinski rules were considered.
3. Considering the Caco2 and Intest Ab. values, it can be said that the absorption will be suitable.
4. The theoretical values of the rate of binding of molecules to plasma proteins (Fu) seem appropriate.
5. The selected molecules also appear not to be skin sensitive.

Conclusion

To sum up, we have found a useful, applicable synthetic approach to yield C-3 substituted pyrazolo[1,5-a]pyrazin-4(5*H*)-one derivatives. Our protocol includes the excess of corresponding amine and Cs_2CO_3, which makes the protocol easier. Primary amine with long alkyl chain and allylamine succeeded the cyclization reaction successively. However, *t*-butyl amine and aniline did not return the cyclic product, which might be due to steric hindrance and poor nucleophilicity. Eleven different pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives were formed, and the reaction mechanism was proposed according to analyzing of reaction media using ^{1}H-NMR. The antiproliferative effect of all synthesized molecules was tested on A549. Compound **5c, 5e, 5f**, and **15e** have shown an antiproliferative effect at a concentration of 160 uM. Theoretical ADMET parameters showed that compounds have good pharmacokinetic parameters, which are considered when a drug candidate was applied to further phase. This approach might be a starting point for extending the synthesis of these types of bicyclic pyrazole derivatives and investigating their further pharmacological effects.

Experimental section

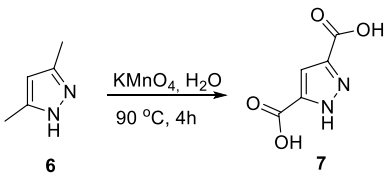
General information

All reagents used were commercially available unless otherwise stated, and all solvents were used as received. Varian 400 spectrometer was used to acquire 400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR spectra. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS) and chloroform-d or d_6-DMSO. HRMS spectra were recorded using by THERMO ITQ900 LC/MS spectrometer with ESI method. All spectroscopic analyses were conducted at the Science Research and Application Center of Van Yuzuncu Yil University.

Synthesis of pyrazole derivatives

Synthesis of starting materials and bicyclic pyrazole derivatives are discussed below.

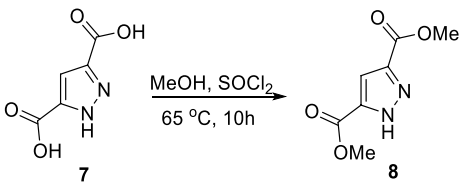
Synthesis of 1H-pyrazole-3,5-dicarboxylic acid [19]



Compound **6**, 0.96 g (10 mmol), was dissolved in 20 mL of water heated to 70 °C, and 7.9 g (50 mmol) of potassium permanganate was added to the hot solution, maintaining the temperature no higher than 90 °C. The mixture was refluxed for 4 h and cooled to room temperature, the precipitate of MnO_2 was filtered off and washed with water, and the filtrate was acidified with aqueous HCl to pH 2 and left overnight. The precipitate was filtered off and washed with water to obtain compound **7** (1 g, 64% yield) as white crystals.

Compound **7**: White crystal, m.p: 286–289 °C (Lit [19].: 289–290 °C).

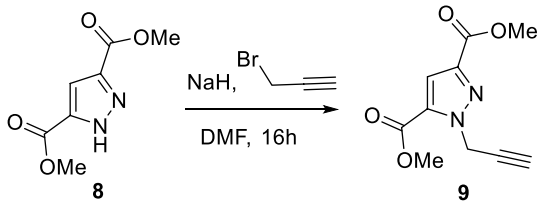
Experimental procedure for the synthesis of dimethyl 1H-pyrazole-3,5-dicarboxylate [20]



Pyrazole-3,5-dicarboxylic acid **7**, (0.56 g, 3.6 mmol) in MeOH (10 mL) at 4 °C was added $SOCl_2$ (1.68 mL, 18 mmol) over 1 h. At the end of the addition, the solution was heated to 60–65 °C for 10 h and then cooled to room temperature. The solution's pH was adjusted to 7.5 with a cold aqueous solution of NaOH, and diester **8** crystallized (0.6 g, 90% yield) as white solid.

Compound **8**: White solid, m.p: 138–140 °C. (Lit [19]: 142–143 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (*s*, 1H, Ar–H), 3.95 (*s*, 6H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.7, 140.2, 111.5, 52.6.

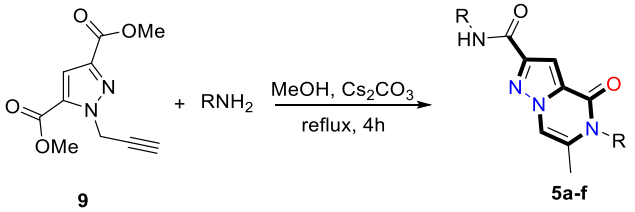
Experimental procedure for the synthesis of dimethyl 1-(prop-2-yn-1-yl)-1*H*-pyrazole-3,5-dicarboxylate (9)



Compound **8** (0.4 g, 2.17 mmol) was dissolved in dimethylformamide (10 mL). NaH (0.09 g, 3.48 mmol, 60% oil suspension) was added to the solution, and the mixture was stirred in an ice bath for 30 min. After completing the reaction, propargyl bromide (0.25 mL, 2.82 mmol) in DMF (5 mL) was added dropwise at room temperature, and the solution was stirred for 16 h. Water was (30 mL) added, and the solution was extracted with ethyl acetate (3 × 15 mL). The combined organic extracts were washed with brine and dried over $MgSO_4$. The solvent was evaporated to give a crude product **9** (0.398 g, 83% yield) as light yellow solid without purification.

Compound **9**: Light yellow solid, m.p: 60–62 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (*s*, 1H, Ar–H), 5.41 (*d*, $J = 2.5$ Hz, 2H, CH_2), 3.92 (bs, 6H, OCH_3), 2.38 (*t*, $J = 2.5$ Hz, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.8, 159.3, 142.8, 133.4, 114.5, 76.7, 74.1, 52.6, 52.4, 42.7. LC/MS: Calculated for $[M+H]^+$ $C_{10}H_{11}N_2O_4^+$ 223.0713, Found 223.0722.

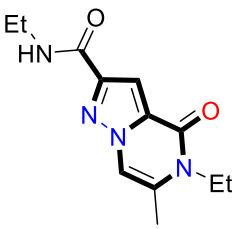
Experimental procedure for the synthesis of pyrazolo[1,5-a] pyrazine-2-carboxamide derivatives (5a–f)



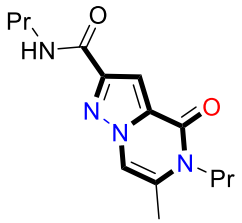
9 5a-f

Compound **9** (0.1 g, 0.45 mmol, 1 eq) was dissolved in MeOH (20 mL). Cesium carbonate (0.146 g, 0.45 mmol, 1 eq) and an excess amount of corresponding amines (5 eq) were added to the solution. The mixture was refluxed for 4 h. Water was (20 mL) added, and the solution was extracted with ethyl acetate (3 × 10 mL). The combined organic extracts were dried over $MgSO_4$. The solvent was evaporated to give a crude product. The crude product was chromatographed on silica gel, eluting with ethyl acetate/hexane (1:1) to give pyrazolo[1,5-a]pyrazine-2-carboxamide derivatives.

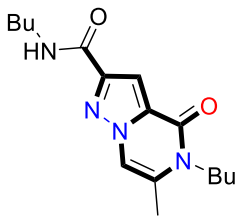
Compound 5a White solid (99 mg, 90% yield), m.p: 192–194 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (bs, 1H, Ar–H), 7.19 (quasi *t*, J = 0.9 Hz, 1H, CH), 6.88 (bs, 1H, NH), 4.05 (*q*, J = 7.1 Hz, 2H, CH_2), 3.52–3.46 (*m*, 2H, CH_2), 2.35 (*d*, J = 1.1 Hz, 3H, CH_3), 1.31 (*t*, J = 7.1 Hz, 3H, CH_3), 1.25 (*t*, J = 7.3 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.1, 155.7, 147.1, 133.6, 128.2, 108.5, 105.8, 38.6, 34.3, 17.2, 15.0, 14.3. HRMS Calculated for $[M+H]^+$ $C_{12}H_{17}N_4O_2^+$ 249.1346, Found 249.1356.



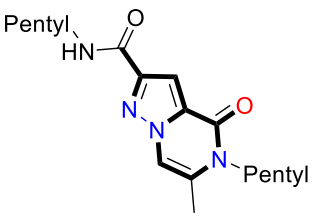
Compound 5b White solid (103 mg, 83%), m.p: 176–178 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (*d*, J = 0.7 Hz, 1H, Ar–H), 7.18 (quasi *t*, J = 0.8 Hz, 1H, CH), 6.94 (*t*, J = 4.9 Hz, 1H, NH), 3.93–3.89 (*m*, 2H, CH_2), 3.43–3.38 (*m*, 2H, CH_2), 2.32 (*d*, J = 1.1 Hz, 3H, CH_3), 1.73–1.60 (*m*, 4H, CH_2), 0.98 (*t*, J = 7.5 Hz, 3H, CH_3), 0.97 (*t*, J = 7.4 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.2, 155.8, 147.1, 133.5, 128.4, 108.4, 105.8, 45.0, 41.1, 23.0, 22.4, 17.3, 11.5, 11.3. HRMS Calculated for $[M+H]^+$ $C_{14}H_{21}N_4O_2^+$ 277.1659, Found 277.1671.



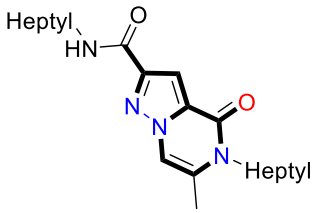
Compound 5c White solid (122 mg, 89%), m.p: 174–176 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (*d*, J = 0.7 Hz, 1H, Ar–H), 7.19 (quasi *t*, J = 0.9 Hz, 1H, CH), 6.89 (*t*, J = 5.5 Hz, 1H, NH), 3.99–3.95 (*m*, 2H, CH_2), 3.48–3.43 (*m*, 2H, CH_2), 2.34 (*d*, J = 1.1 Hz, 3H, CH_3), 1.69–1.59 (*m*, 4H, CH_2), 1.47–1.37 (*m*, 4H, CH_2), 0.98 (*t*, J = 7.4 Hz, 3H, CH_3), 0.95 (*t*, J = 7.4 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.2, 155.8, 147.2, 133.6, 128.3, 108.5, 105.9, 43.5, 39.2, 31.9, 31.2, 29.9, 29.8, 20.4, 20.3, 17.4. HRMS Calculated for $[M+H]^+$ $C_{16}H_{25}N_4O_2^+$ 305.1972, Found 305.1983.



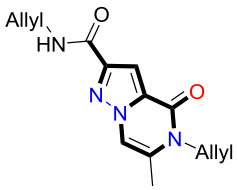
Compound 5d White solid (125.6 mg, 84%), m.p: 132–135 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (*d*, J = 0.6 Hz, 1H, Ar–H), 7.19 (quasi *t*, J = 0.9 Hz, 1H, CH), 6.91 (*t*, J = 5.7 Hz, 1H, NH), 3.96–3.92 (*m*, 2H, N–CH_2), 3.46–3.40 (*m*, 2H, N–CH_2), 2.33 (*d*, J = 1.1 Hz, 3H, CH_3), 1.38–1.27 (*m*, 12H, CH_2), 0.91–0.85 (*m*, 6H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.2, 155.8, 147.0, 133.6, 128.4, 108.5, 105.9, 43.7, 39.4, 29.5, 29.2, 29.1, 28.8, 22.5, 22.4, 17.4, 14.1, 14.0. HRMS Calculated for $[M+H]^+$ $C_{18}H_{29}N_4O_2^+$ 333.2285, Found 333.2297.



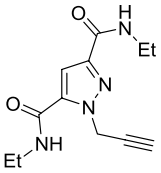
Compound 5e White solid (150.3 mg, 86%), m.p: 143–145 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (*d*, $J=0.7$ Hz, 1H, Ar–H), 7.19 (quasi *t*, $J=0.9$ Hz, 1H, CH), 6.91 (*t*, $J=5.9$ Hz, 1H, NH), 3.96–3.92 (*m*, 2H, N–CH_2), 3.46–3.40 (*m*, 2H, N–CH_2), 2.33 (*d*, $J=1.1$ Hz, 3H, CH_3), 1.70–1.57 (*m*, 4H, CH_2), 1.38–1.24 (*m*, 16H, CH_2), 0.88–0.85 (*m*, 6H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.1, 155.8, 147.1, 133.6, 128.3, 108.5, 105.9, 43.7, 39.5, 31.9, 31.8, 29.8, 29.1, 29.1, 27.1, 27.0, 22.7, 22.6, 17.4, 14.2, 14.1. HRMS Calculated for $[M+H]^+$ $C_{22}H_{37}N_4O_2^+$ 389.2911, Found 389.2927.



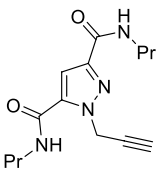
Compound 5f White solid (106 mg, 87%), m.p: 161–164 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (*d*, $J=0.7$ Hz, 1H, Ar–H), 7.22 (quasi *t*, $J=0.9$ Hz, 1H, CH), 7.02 (*t*, $J=5.5$ Hz, 1H, NH), 5.98–5.88 (*m*, 2H, =CH), 5.30–5.21 (*m*, 2H, $=CH_2$), 5.18 (dq, $J=1.4$ and 10.2 Hz 1H, $=CH_2$), 5.10 (dtd, $J=0.8$, 1.8 and 17.2 Hz 1H, $=CH_2$), 4.66 (dt, $J=1.7$, 4.9 Hz, 2H, N–CH_2), 4.09 (tt, $J=1.6$, 5.9 Hz, 2H, N–CH_2), 2.33 (*d*, $J=1.1$ Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.0, 155.6, 146.8, 134.1, 133.4, 132.1, 128.8, 117.2, 116.7, 108.5, 106.4, 45.2, 41.8, 17.1. HRMS Calculated for $[M+H]^+$ $C_{14}H_{17}N_4O_2^+$ 273.1346, Found 273.1356.



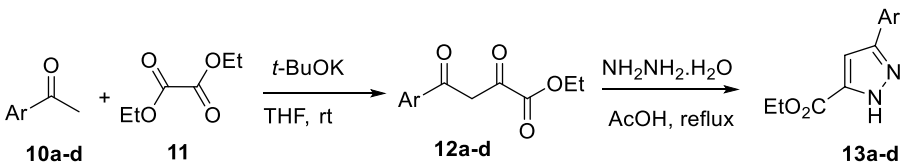
Compound 10a White solid (71 mg, 70% yield), m.p: 154–156 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (*s*, 1H, Ar–H), 7.05 (quasi *t*, $J=3.7$ Hz, 1H, NH), 6.94 (quasi *t*, $J=4.4$ Hz, 1H, NH), 5.46 (*d*, $J=2.5$ Hz, 2H, CH_2), 3.51–3.42 (*m*, 4H, CH_2), 2.39 (*t*, $J=2.5$ Hz, 1H, CH), 1.25 (*t*, $J=7.2$ Hz, 3H, CH_3), 1.25 (*t*, $J=7.3$ Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.3, 159.0, 145.7, 137.0, 107.7, 77.7, 73.5, 41.8, 34.8, 34.3, 15.0, 14.9. HRMS Calculated for $[M+H]^+$ $C_{12}H_{17}N_4O_2^+$ 249.1346, Found 249.1356.



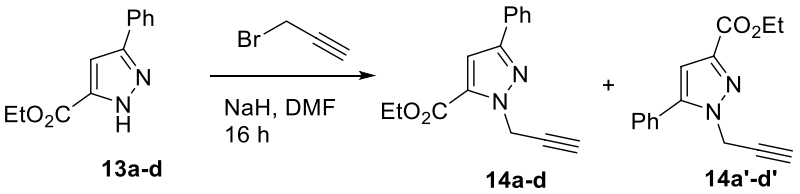
Compound 10b White solid (71.5 mg, 65%), m.p: 107–109 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (*t*, $J=5.7$ Hz, 1H, NH), 7.54 (*s*, 1H, Ar–H), 7.02 (*t*, $J=5.8$ Hz, 1H, NH), 5.47 (*d*, $J=2.5$ Hz, 2H, CH_2), 3.40–3.33 (*m*, 4H, CH_2), 2.38 (*t*, $J=2.5$ Hz, 1H, CH), 1.66–1.60 (*m*, 4H, CH_2), 0.97 (*t*, $J=7.4$ Hz, 3H, CH_3), 0.95 (*t*, $J=7.4$ Hz, 3H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.4, 159.1, 145.7, 137.0, 107.7, 77.6, 73.6, 41.8, 41.5, 41.1, 23.0, 11.6, 11.5. HRMS Calculated for $[M+H]^+$ $C_{14}H_{21}N_4O_2^+$ 277.1659, Found 277.1670.



Experimental procedure for the synthesis of ethyl 3-phenyl-1-(prop-2-yn-1-yl)-1H-pyrazole-5-carboxylate and ethyl 5-phenyl-1-(prop-2-yn-1-yl)-1H-pyrazole-3-carboxylate (14a–d)



10a-d **11** **12a-d** **13a-d** **(1)** (1)



(2)

Reaction 1 [22]: A solution of *t*-BuOK (36 mmol) in THF (30 mL) was added to a mixture of acetophenone derivatives **10a–d** (26 mmol) and diethyl oxalate (**11**) (28 mmol). The final mixture was stirred at room temperature for 15 h to form **12a–d**. Thereafter, AcOH (64 mmol) and hydrazine hydrate (48 mmol) were added. The mixture was heated under reflux for 2 h. The mixture was evaporated in a vacuum after being cooled, and the residue was extracted with ethyl acetate (50 mL). The organic phase was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated with reduced pressure.

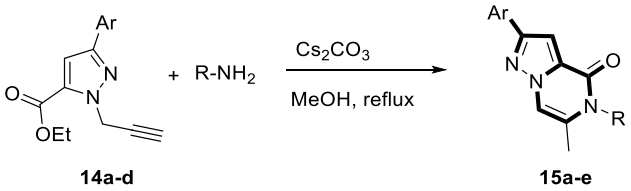
Reaction 2: Compound **13a–d** (1 g, 4.63 mmol) was dissolved in dimethylformamide (10 mL). NaH (0.22 g, 9.26 mmol, 60% oil suspension) was added to the solution, and the mixture was stirred in an ice bath for 30 min. After completing the reaction, propargyl bromide (0.56 ml, 6.95 mmol) in DMF (5 mL) was added dropwise at room temperature, and the solution was stirred for 16 h. Water was (30 mL) added, and the solution was extracted with ethyl acetate (3 × 15 mL). The combined organic extracts were washed with brine and dried over $MgSO_4$. The solvent was evaporated to give crude products **14a–d** and **14a′–d′** as a compound mixture.

Note: Propargylation reaction gave two different isomers. One of these isomers cannot be used for cyclization (**14a′-d′**). However, to see the exact propargylated molecules, our target starting materials, only one mixture was purified. Therefore, the others were used as a mixture of two isomers, and the purification was done after cyclization reaction.

Compound **14a**: Light yellow solid, m.p: 75–77 °C, 65%. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.83–7.80 (*m*, 2H, Ar–H), 7.43–7.39 (*m*, 2H, Ar–H), 7.35–7.31 (*m*, 1H, Ar–H), 7.17 (*s*, 1H, Pyr-H), 5.41 (*d*, J = 2.5 Hz, 2H, -NCH_2), 4.40 (*q*, J = 7.2 Hz, 2H, -OCH_2), 2.38 (*t*, J = 2.5 Hz, 1H, –CH), 1.42 (*t*, J = 7.2 Hz, 3H, –CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 159.4, 150.8, 133.3, 132.2, 128.7, 128.2, 125.7, 108.6, 77.7, 73.1, 61.4, 41.6, 14.2.

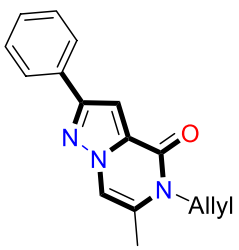
Compound **14a′**: Yellow crystal, m.p: 93–95 °C, 45%. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.50–7.47 (*m*, 2H, Ar–H), 7.46–7.40 (*m*, 3H, Ar–H), 6.82 (*s*, 1H, Pyr-H), 4.90 (*d*, J = 2.5 Hz, 2H, -NCH_2), 4.37 (*q*, J = 7.1 Hz, 2H, -OCH_2), 2.42 (*t*, J = 2.5 Hz, 1H, –CH), 1.35 (*t*, J = 7.1 Hz, 3H, –CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 162.2, 145.1, 143.7, 129.3, 129.1, 129.0, 128.8, 109.1, 77.4, 74.4, 61.1, 40.6, 14.4.

Experimental procedure for the synthesis of pyrazolo[1,5-a] pyrazin-4(5*H*)-one derivatives (15a–e)

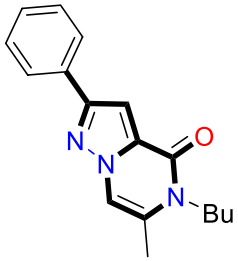


The mixture of Compound **14a–d and 14a′–d′** (4 mmol) was dissolved in MeOH (20 mL). Cesium carbonate (4 mmol) and an excess amount of corresponding amines (20 mmol) were added to the solution. The mixture was refluxed for 4 h. Water was (20 mL) added, and the solution was extracted with ethyl acetate (3 × 10 mL). The combined organic extracts were dried over $MgSO_4$. The solvent was evaporated to give a crude product. The crude product was purified with column chromatography on silica gel, eluting with ethyl acetate/hexane (1:3) to give pyrazolo[1,5-a] pyrazin-4(5*H*)-one derivatives (15a–e).

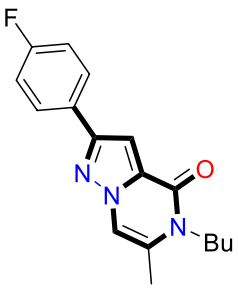
Allyl amine was used. Compound 15a White solid (0.55 g, 88% yield), m.p: 120–122 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.90–7.87 (*m*, 2H, Ar–H), 7.46–7.42 (*m*, 2H, Ar–H), 7.38–7.34 (*m*, 1H, Ar–H), 7.33 (bs, 1H, =CH), 7.31 (*s*, 1H, Ar–H), 5.95 (ddt, J = 4.9, 9.9, 15.3 Hz, 1H, =CH), 5.25–5.21 (*m*, 1H, =CH_2), 5.13–5.08 (*m*, 1H, =CH_2), 4.68 (dt, J = 1.7, 4.9 Hz, 2H, N–CH_2), 2.32 (*d*, J = 1.0 Hz, 3H, –CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 155.7, 152.4, 133.2, 132.3, 132.2, 128.8, 128.5, 126.4, 126.1, 116.7, 108.9, 101.8, 44.9, 16.9.



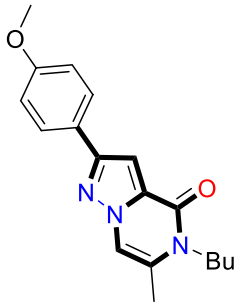
Butyl amine was used. Compound 15b White solid (0.57 g, 86% yield), m.p: 86–88 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.89–7.86 (*m*, 2H, Ar–H), 7.45–7.40 (*m*, 2H, Ar–H), 7.37–7.32 (*m*, 1H, Ar–H), 7.30–7.29 (*m*, 1H, =CH), 7.27 (*d*, J = 0.7 Hz, 1H, Ar–H), 4.00–3.96 (*m*, 2H, N–CH_2), 2.32 (*d*, J = 1.0 Hz, 3H, –CH_3), 1.72–1.64 (*m*, 2H, –CH_2), 1.48–1.39 (*m*, 2H, –CH_2), 0.98 (*t*, J = 7.4 Hz, 3H, –CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 155.9, 152.3, 133.4, 132.4, 128.8, 128.4, 126.1, 126.0, 108.8, 101.4, 43.2, 31.1, 30.9, 20.2, 17.2, 13.8.



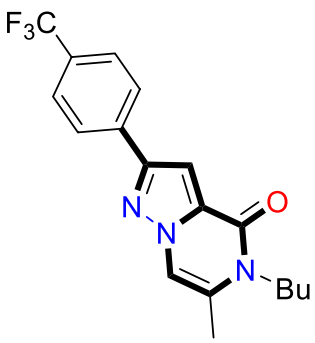
Butyl amine was used. Compound 15c White solid (0.62 g, 94% yield), m.p: 159–160 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.86–7.82 (*m*, 2H, Ar–H), 7.29–7.28 (*m*, 1H, =CH), 7.21 (*s*, 1H, Ar–H), 7.14–7.09 (*m*, 2H, Ar–H), 4.01–3.97 (*m*, 2H, N–CH_2), 2.34 (*d*, J = 0.9 Hz, 3H, –CH_3), 1.72–1.64 (*m*, 2H, –CH_2), 1.48–1.39 (*m*, 2H, –CH_2), 0.98 (*t*, J = 7.4 Hz, 3H, –CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 162.9 (*d*, J = 247.7 Hz), 155.8, 151.4, 133.5, 128.6 (*d*, J = 3.1 Hz), 127.8 (*d*, J = 8.2 Hz), 126.2, 115.7 (*d*, J = 21.7 Hz), 108.8, 101.2, 43.2, 31.1, 20.2, 17.2, 13.8.



Butyl amine was used. Compound 15d White solid (0.55 mg, 84% yield), m.p: 128–130 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.82–7.79 (AA′BB′, 2H, Ar–H), 7.29–7.28 (*m*, 1H, =CH), 7.19 (*d*, J = 0.7 Hz, 1H, Ar–H), 6.98–6.94 (AA′BB′, 2H, Ar–H), 4.00–3.96 (*m*, 2H, N–CH_2), 3.84 (*s*, 3H, -OCH_3), 2.33 (*d*, J = 1.1 Hz, 3H, –CH_3), 1.72–1.64 (*m*, 2H, –CH_2), 1.48–1.39 (*m*, 2H, –CH_2), 0.98 (*t*, J = 7.4 Hz, 3H, –CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 159.9, 155.8, 152.2, 133.4, 127.3, 125.7, 125.1, 114.2, 108.8, 100.8, 55.3, 43.2, 31.1, 20.2, 17.2, 13.8.



Butyl amine was used. Compound 15e White solid (0.585 g, 90% yield), m.p: 171–173 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.00–7.98 (AA′BB′, 2H, Ar–H), 7.70–7.67 (AA′BB′, 2H, Ar–H), 7.32 (*s*, 1H, Ar–H), 7.32–7.31(*m*, 1H, =CH), 4.02–3.98 (*m*, 2H, N–CH_2), 2.36 (*d*, J = 1.0 Hz, 3H, –CH_3), 1.73–1.65 (*m*, 2H, –CH_2), 1.49–1.40 (*m*, 2H, –CH_2), 0.99 (*t*, J = 7.4 Hz, 3H, –CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 155.8, 150.7, 135.8, 133.6, 130.2, 130.0, 126.8, 126.2, 125.7 (*q*, J = 3.8 Hz, –CF_3), 108.7, 102.0, 43.2, 31.1, 20.2, 17.2, 13.8.



Cell viability assay (MTT)

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in DMEM at 5 × 103 cells/200 µl per well in 96-well plates for 24 h at 37 °C. When cells were attached after 24 h, cells were treated with DMSO (control group) and Pyrazolo[1,5-a]pyrazin-4(5*H*)-one derivatives (5 µM, 10 µM, 20 µM, 40 µM, 80 µM, and 160 µM) for 24 and 48 h. After incubation time, 10 µL MTT solution (5 mg/mL) was added to each well. After 4 h of MTT incubation at

37 °C, 100 µL crystal dissolving buffer was added, and the plates were gently shaken on an orbital shaker for 5 min. The absorbance at 570 nm was measured with the SpectraMax ID3 microplate reader. Each treatment was repeated 4 times. The mean absorbance of four wells was used as an indicator of relative cell growth.

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