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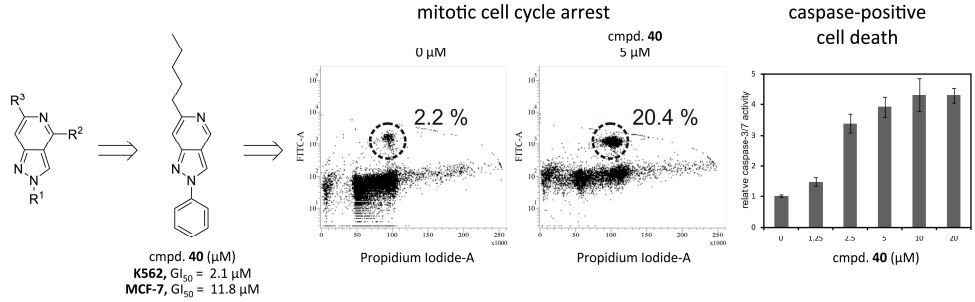

cmpd. **40** (µM)
K562, GI_{50} = 2.1 µM
MCF-7, GI_{50} = 11.8 µM

Synthesis and anti-mitotic activity of 2,4- or 2,6-disubstituted- and 2,4,6-trisubstituted-2*H*-pyrazolo[4,3-*c*]pyridines

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Abstract

An efficient synthetic route for the synthesis of 2*H*-pyrazolo[4,3-*c*]pyridines, primarily varying by the substituents at the 2-, 4- and 6-positions, is described here. A Sonogashira-type cross-coupling reaction was employed to yield 3-alkynyl-1*H*-pyrazole-4-carbaldehydes, ethanones and propanones from the corresponding 1*H*-pyrazol-3-yl trifluoromethanesulfonates. Subsequent treatment of the coupling products with dry ammonia afforded a versatile library of 2*H*-pyrazolo[4,3-*c*]pyridines, which were then evaluated for their cytotoxicity against K562 and MCF-7 cancer cell lines. The most potent of these compounds displayed low micromolar GI_{50} values in both cell lines. Active compounds induced dose-dependent cell-cycle arrest in mitosis, as shown by flow cytometric analysis of DNA content and phosphorylation of histone H3 at serine-10. Moreover, biochemical assays revealed increased activities of caspases-3/7 in treated cells, specific fragmentation of PARP-1, and phosphorylation of Bcl-2, collectively confirming apoptosis as the mechanism of cell death.

Keywords:

apoptosis; G2/M cell cycle arrest; pyrazole; structure-activity relationships

1. Introduction

Due to its wide spectrum of biological activities, pyrazole is a common structural unit in many pharmaceuticals [1] and a central axis of numerous ongoing studies devoted to the synthesis and biological evaluation of novel pyrazole moiety-bearing molecules [2-9]. Among the vast variety of biologically active annelated pyrazole derivatives [10, 11], synthetically demanding 2*H*-pyrazolo[4,3-*c*]pyridines are relatively understudied. Scarce examples of such biologically active compounds (Fig. 1) include 3-amino-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine-4,6-diol, which has been revealed to act as a p90 ribosomal S6 kinase 2 (RSK2) inhibitor [12], or 2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-3-amine, N^4-(2-methoxyethyl)-6-methyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine-3,4-diamine and 6-methyl-2-phenyl-4-(piperidin-1-yl)-2*H*-pyrazolo[4,3-*c*]pyridin-3-amine, which have been evaluated as inhibitors against a wide range of kinases, revealing their moderate potency against p38α, aurora A and CK1δ, respectively [13].

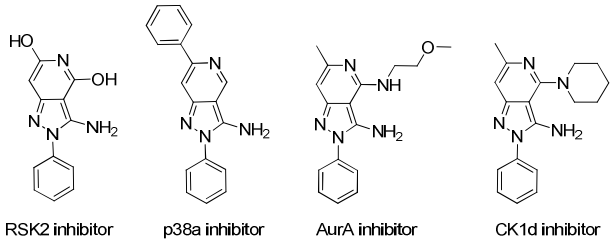


Fig. 1. Examples of biologically relevant 2*H*-pyrazolo[4,3-*c*]pyridines

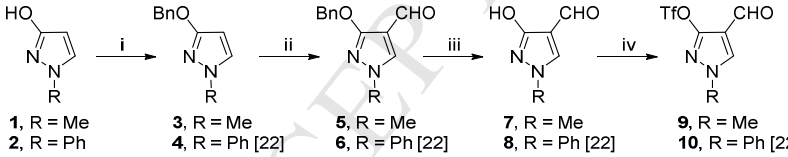
The synthesis of 2*H*-pyrazolo[4,3-*c*]pyridines is usually accomplished by the treatment of 4-chloro or 4-iodopyridines possessing a carbonyl moiety at the 3-position with various hydrazines [14-19] at room or elevated temperatures. Similarly, 4-chloronicotinonitriles react with hydrazines in the presence of organic bases forming 2-substituted-2*H*-pyrazolo[4,3-*c*]pyridines [18, 20]. A series of 2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridines were also obtained by heating mixtures of (*E*)-1-(4-azidopyridin-3-yl)-*N*-phenylmethanimines in toluene at 105 °C [21]. On the other hand, we have recently demonstrated that 2*H*-pyrazolo[4,3-*c*]pyridines can also be easily accessed from 3-hydroxy-1-phenyl-1*H*-pyrazole-4-carbaldehyde and the corresponding ethanone *via* intermediate triflates making use of a Sonogashira cross-coupling and dry ammonia induced cyclization reactions [22]. Therefore, in this study, we further examined the applicability of our synthetic approach and prepared a library of various 2*H*-pyrazolo[4,3-*c*]pyridines, primarily varying by the substituents at the 2-, 4- and 6-positions in order to assess their biological activity and to formulate possible structure-activity relationships.

2. Results and Discussion

2.1. Synthesis

In a series of recent publications we have demonstrated that pyrazole-4-carbaldehydes carrying an alkynyl function adjacent to the formyl moiety are valuable starting materials for the construction of condensed pyrazole systems, for instance: dipyrazolo[1,5-*a*:4,3-*c*]pyridines [23], 2*H*- and 3*H*-pyrazolo[4',3':3,4]pyrido[1,2-*a*]benzimidazoles, 3*H*-pyrazolo[4,3-*c*]imidazo[1,2-*a*:5,4-*b*']dipyridines and 13,13a-dihydro-3*H*-pyrazolo[4',3':3,4]pyrido[1,2-*a*]pyrimidines [24] and 2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridines [25, 26].

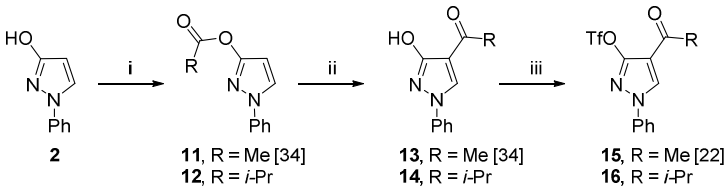
In this study, we further elaborated the synthetic potential of 1-substituted-1*H*-pyrazoles carrying an alkynyl function adjacent to the carbonyl moiety. The synthesis of starting triflates **9**, **10**, **15**, and **16** for the Sonogashira cross-coupling reaction is presented in Schemes 1 and 2. Commercially available but otherwise undescribed 3-hydroxy-1-methyl-1*H*-pyrazole-4-carbaldehyde **7**, the precursor for the preparation of the triflate **9**, was synthesized following an analogous approach, which we have previously described for its analog 3-hydroxy-1-phenyl-1*H*-pyrazole-4-carbaldehyde **8** [22] (Scheme 1). In short, we applied the Vilsmeier-Haack reaction conditions for the synthesis of 4-benzyloxy-1-methyl-1*H*-pyrazole **3**, which was obtained by benzylation of the readily available starting compound **1** [25] under standard conditions with benzyl chloride, similarly to the described procedure [27, 28]. Upon heating compound **3** with DMF/$POCl_3$ at 70 °C for half an hour the target carbaldehyde **5** was formed in 60% yield. Alternatively, the preparation of carbaldehyde **5** can also be achieved by the oxidation of (3-(benzyloxy)-1-methyl-1*H*-pyrazol-4-yl)methanol with manganese dioxide [29]. Debenzylation of compound **5** was accomplished by treatment with TFA in toluene, conditions typically used for the selective deprotection of *O*-benzylsalicylaldehydes [30], furnishing the target carbaldehyde **7** in 90% yield. Having precursor **7** in hand, we further treated it with triflic anhydride in the presence of TEA in DCM [31] to obtain the corresponding triflate **9** in 85% yield. The same synthetic pathway was applied to obtain 3-triflyloxy-1-phenyl-1*H*-pyrazole-4-carbaldehyde **10** from 3-hydroxy-1-phenyl-1*H*-pyrazole **2**, as we have described previously [22].



Scheme 1. *Reagents and conditions:* (i) BnCl, NaH, DMF, argon atmosphere, 0-60 °C, 1 h, 90% (for **3**), 85% (for **4**); (ii) $POCl_3$, DMF, 0-60 °C, 0.5 h, 85% (for **5**), 81% (for **6**); (iii) TFA, toluene, rt, 15 h, 90% (for **7**), 85% (for **8**); (iv) Tf_2O, TEA, DCM, rt, 1 h, 85% (for **9**), 83% (for **10**).

Triflates **15** and **16** were synthesized similarly to the previously described approach [22] (Scheme 2). Fries rearrangement conditions ($AlCl_3$, SC_2) were applied to 1-phenyl-1*H*-pyrazol-3-yl acetate **11** [32] and the corresponding pivalate **12**, which were obtained from readily

available 3-hydroxy-1-phenyl-1*H*-pyrazole **2** [33] and acetic anhydride or isobutylchloride, respectively. Triflation of 1-phenyl-1*H*-pyrazol-3-ols **13** [34] and **14** afforded 1-phenyl-1*H*-pyrazol-3-yl trifluoromethanesulfonates **15** and **16** in 85% and 96% yield, respectively.

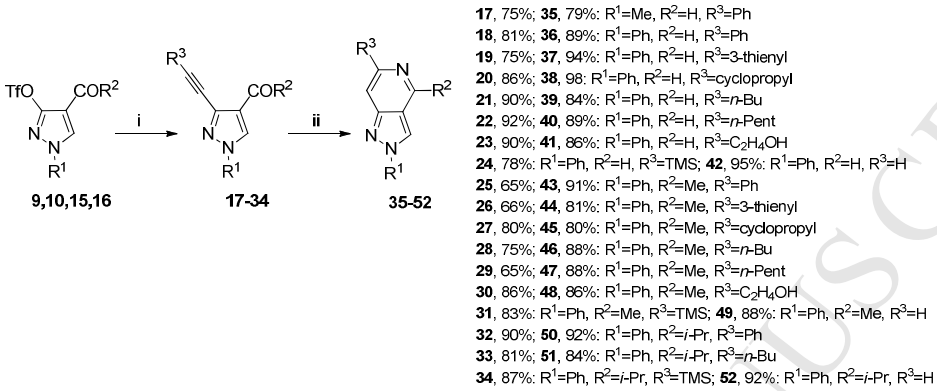


Scheme 2. *Reagents and conditions:* (i) Ac_2O, 100 °C, 0.5 h, 79% (for **11**); isobutylchloride, pyridine, DCM, rt, 1 h, 90% (for **12**); (ii) $AlCl_3$, CS_2, reflux, 3 h, 74% (for **13**), 89% (for **14**); (iii) Tf_2O, TEA, DCM, rt, 1 h, 85% (for **15**), 96% (for **16**).

The construction of the target pyrazolopyridines from 3-alkynylpyrazole-4-carbaldehydes, ethanones and propanones is represented in Scheme 3. The prepared triflates **9**, **10**, **15** and **16** were successfully coupled with various alkyl and aryl acetylenes under standard Sonogashira cross-coupling reaction conditions ($Pd(PPh_3)_2Cl_2$, CuI, TEA, DMF) to give the corresponding 3-alkynyl-1-phenyl-1*H*-pyrazoles **17**-**34** in good to very good yields, in most cases (Scheme 3). Couplings of 4-acetyl-1-phenyl-1*H*-pyrazol-3-yl trifluoromethanesulfonate (**15**) with phenyl-, 3-thienyl- and *n*-pentylacetylenes gave corresponding products in fair yields of less than 70%. Notably, 1-phenyl-3-triflyl-1*H*-pyrazole-4-carbaldehydes resulted in better yields of the Sonogashira cross-coupling reaction in comparison with the corresponding 4-ethanones. Finally, compounds **17**-**34** bearing the alkynyl group moiety adjacent to the carbonyl group were treated with dry ammonia under elevated temperature and pressure, allowing the direct formation of pyrazolo[4,3-*c*]pyridines **35**-**52** generally in very good to excellent yields.

It is noteworthy that this methodology proved to be applicable for the synthesis of 2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine **42** and its 4-methyl and 4-isopropyl derivatives, **49** and **52**, respectively. Synthesis of the aforementioned **42** has been accomplished before, employing inconvenient highly toxic and unstable reactants, i.e., by cycloaddition of 3-pyridyne, which is accessible by lead tetraacetate oxidation of 1-aminotriazolo[4,5-*c*]pyridine to *N*-phenylsydnone [35]. In contrast, our synthetic approach makes use of the convenient TMS-protected, commercially available, trimethylsilylacetylene, which efficiently undergoes Sonogashira cross-coupling with

corresponding triflates **10, 15,** and **16**, furnishing **24**, **31**, and **34** in 78-87% yield. The latter, due to the convenient lability of TMS protecting group, upon treatment with dry ammonia under elevated temperature and pressure directly gives rise to the target TMS-deprotected 2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridines **42**, **49** and **52** in 88-95% yield (Scheme 3).



17, 75%; **35**, 79%: R^1=Me, R^2=H, R^3=Ph
18, 81%; **36**, 89%: R^1=Ph, R^2=H, R^3=Ph
19, 75%; **37**, 94%: R^1=Ph, R^2=H, R^3=3-thienyl
20, 86%; **38**, 98: R^1=Ph, R^2=H, R^3=cyclopropyl
21, 90%; **39**, 84%: R^1=Ph, R^2=H, R^3=*n*-Bu
22, 92%; **40**, 89%: R^1=Ph, R^2=H, R^3=*n*-Pent
23, 90%; **41**, 86%: R^1=Ph, R^2=H, R^3=C_2H_4OH
24, 78%: R^1=Ph, R^2=H, R^3=TMS; **42**, 95%: R^1=Ph, R^2=H, R^3=H
25, 65%; **43**, 91%: R^1=Ph, R^2=Me, R^3=Ph
26, 66%; **44**, 81%: R^1=Ph, R^2=Me, R^3=3-thienyl
27, 80%; **45**, 80%: R^1=Ph, R^2=Me, R^3=cyclopropyl
28, 75%; **46**, 88%: R^1=Ph, R^2=Me, R^3=*n*-Bu
29, 65%; **47**, 88%: R^1=Ph, R^2=Me, R^3=*n*-Pent
30, 86%; **48**, 86%: R^1=Ph, R^2=Me, R^3=C_2H_4OH
31, 83%: R^1=Ph, R^2=Me, R^3=TMS; **49**, 88%: R^1=Ph, R^2=Me, R^3=H
32, 90%; **50**, 92%: R^1=Ph, R^2=*i*-Pr, R^3=Ph
33, 81%; **51**, 84%: R^1=Ph, R^2=*i*-Pr, R^3=*n*-Bu
34, 87%: R^1=Ph, R^2=*i*-Pr, R^3=TMS; **52**, 92%: R^1=Ph, R^2=*i*-Pr, R^3=H

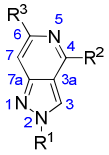
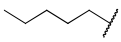
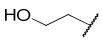
Scheme 3. *Reagents and conditions*: (i) R_3-C≡CH, $Pd(PPh_3)_2Cl_2$, TEA, CuI, DMF, 60 °C, 1-38 h; (ii) NH_3, MeOH, 120 °C, 15 h.

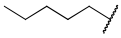
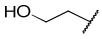
2.2. Anticancer activity *in vitro*

Prepared compounds **35-52** were evaluated for their cytotoxicity against two human cancer cell lines: K562 (chronic myeloid leukemia cells) and MCF-7 (breast cancer cells). In general, most tested compounds exhibited moderate cytotoxicity, with GI_{50} values in the micromolar range (Table 1). The most potent derivatives bear a phenyl moiety at the 2-position, with no substituent at the 4-position and either an aryl or alkyl at the 6-position (compounds **36**, **37**, **39**, **40**). It is noteworthy that replacing a phenyl substituent at the 2-position (compound **36**) with a methyl group resulted in a complete loss of the activity (compound **35**). Increasing the bulkiness of the substituents at the 4-position reduced cytotoxicity of the derivatives, i.e., introducing methyl (compounds **43**, **47**) and isopropyl (compound **50**) substituents resulted in a gradual decrease in potency of the compounds compared to unsubstituted derivatives (compounds **36**, **40**). Replacement of the substituents at the 6-position enabled the fine-tuning of cytotoxicity of the compounds. Surprisingly, a lack of the substituent at this position or introduction of a polar ethylhydroxy group resulted in completely inactive derivatives (compounds **41**, **42**, **48**, **49**), while various other substituents were relatively well tolerated. The most effective substituents at 6-position were alkyl chains and aromatic rings. Derivatives bearing a phenyl ring at the 7-

position exhibited good cytotoxic values, with GI_{50} values for compound **36** reaching 3.4 μM for K562 and 4.8 μM for MCF-7 cells. Unfortunately, attempts to replace the phenyl ring with a slightly less aromatic thiophene or aliphatic cyclopropyl rings resulted in less active derivatives; phenyl derivatives **36** and **43** were twofold more potent than analogous thiophen-3-yl derivatives **37** and **44**. Interestingly, replacing a cyclopropyl ring with linear aliphatic substituents resulted in an increased cytotoxicity of the compounds. Thus, out of the prepared derivatives, compound **40**, bearing a pentyl substituent at the 6-position, a phenyl ring at the 2-position and lacking a substituent at the 4-position, proved to be the most cytotoxic, reaching a GI_{50} value of 2.1 μM for K562 cells.

Table 1. *In vitro* cytotoxicity of previously and newly synthesized 2*H*-pyrazolo[4,3-*c*]pyridines **35-52**.

General structure	Compound	R^1	R^2	R^3	GI_{50} (μM)*	
					K562	MCF-7
 **35-52**	**35**	Me	H		>100	>100
	36		H		3.4	4.8
	37		H		8.3	9.8
	38		H		60.5	69.3
	39		H		6.7	13.3
	40		H		2.1	11.8
	41		H		>100	>100
	42		H	H	>100	>100
	43		Me		6.3	11.0
	44		Me		10.0	22.2

45		Me		25.9	63.5
46		Me		8.5	11.1
47		Me		6.9	17.0
48		Me		>100	>100
49		Me	H	>100	>100
50		*i*-Pr		36.8	91.0
51		*i*-Pr		12.9	33.3
52		*i*-Pr	H	>100	98.8

* Data are means of at least two independent measurements.

2.3. Effect on the cell cycle and apoptosis

All compounds displaying a GI_{50} lower than 80 µM in at least one cell line underwent cell-cycle investigation in K562 and MCF-7 cell lines to gain preliminary information about their mechanism of action. The cells were treated with the tested compounds for 24 hours at 10 µM concentrations. Tubuline-interfering agent nocodazole and cyclin-dependent kinase 4 inhibitor palbociclib were used as positive controls, causing dominant mitotic and G1 arrest, respectively. The majority of compounds exhibited a clear effect on the cell cycle in both cell lines; with increased cell populations in G2/M phases and corresponding decreases in G1 and S phase populations observed post treatment (Table 2). The highest percentages of a G2/M population were found in cultures treated with compounds **36**, **39**, **40**, **43** and **47**, all of which also displayed the strongest cytotoxicities (Table 1). In addition, a substantial increase of sub-G1 populations was observed in cultures treated with the most potent compounds **36**, **37**, **39**, **40**, **43**, **44**, **46** and **51**, indicating ongoing apoptosis. Sub-G1 populations were usually higher in K562 cells, probably as a consequence of their higher sensitivity to novel compounds, which was observed in the cytotoxicity assays (Table 1).

Table 2. Cell cycle analysis in K562 and MCF-7 cells treated with active 2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridines at a single dose of 10 μM.

Compound	**K562 cell cycle phases (%)**				**MCF-7 cell cycle phases (%)**			
	subG1	**G1**	**S**	**G2/M**	**subG1**	**G1**	**S**	**G2/M**
Untreated	15.0	34.6	50.3	15.0	7.1	60.7	34.1	5.3
36	43.0	19.8	5.5	74.7	14.3	56.8	26.2	17.0
37	34.2	21.7	37.3	41.1	24.2	39.5	29.1	31.4
38	19.6	32.3	52.2	15.5	7.5	64.7	29.4	5.9
39	37.7	24.8	12.8	62.3	31.1	36.0	30.3	33.7
40	35.4	14.8	3.3	82.0	50.3	18.1	26.3	55.6
43	46.2	13.7	36.1	50.2	11.9	65.5	20.5	14.1
44	45.7	26.2	29.2	44.6	22.7	37.2	31.2	31.6
45	15.3	34.7	45.8	19.6	9.0	63.6	30.2	6.3
46	40.8	28.2	30.5	41.4	19.3	48.5	24.0	27.5
47	28.3	21.8	28.3	49.9	31.1	50.2	18.1	31.7
50	21.3	35.1	46.6	18.3	6.7	62.6	30.6	6.9
51	47.1	26.7	44.2	29.0	7.8	66.1	24.5	9.4
nocodazole (25 ng/mL)	49.4	3.6	26.8	69.7	32.1	14.4	49.9	35.7
palbociclib (0.25 μM)	35.0	54.0	30.1	15.9	7.5	90.0	5.5	4.6

Compound **40**, with the strongest effect on the cell cycle, was further assayed at several concentrations (Fig. 2a). DNA histograms revealed a clear dose-dependent arrest in G2/M phases. To discriminate between arrest in G2 and M, we quantified the phosphorylation of histone H3 at serine-10, a common mitotic marker. Flow cytometric analysis revealed a strong accumulation of cells with phosphorylated histone, confirming arrest in mitosis (Fig. 2b).

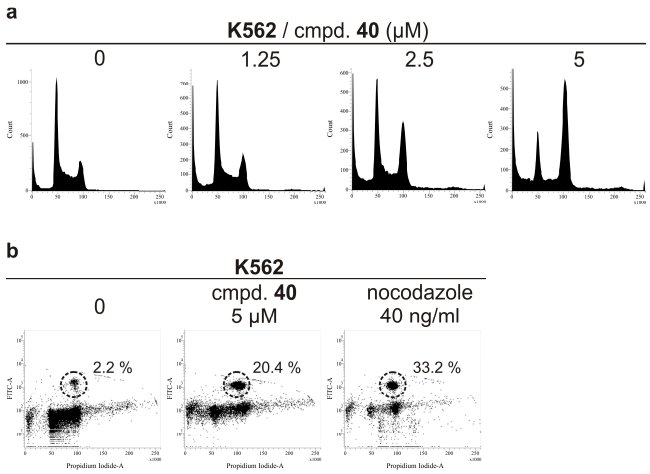


Fig. 2. Cell cycle arrest in K562 cells treated with compound **40** for 24 h. (a) DNA histograms of cells treated with different doses of the compound **40**. (b) Phosphorylation of histone H3 at serine-10 in cells treated with 5 μM dose of compound **40**. Nocodazole was used as a positive control.

Due to the strong cytotoxicity of **40** in the K562 cell line, we sought to identify the type of cell death that occurs. Caspase activation in treated cells was measured by an enzymatic assay, using the fluorescently labeled peptide substrate Ac-DEVD-AMC of caspases 3 and 7 (Fig. 3a) which revealed a clear dose-dependent responses in the micromolar range. In parallel, lysates of treated K562 and MCF-7 cells were subjected to immunoblotting, the analysis of which revealed a dose-dependent increase in the 89 kDa fragment of PARP-1, a known caspase substrate (Fig. 3b). Phosphorylation of histone H2AX at Ser-139 (γH2AX), a modification required for DNA fragmentation during apoptosis [36], was also detected. Additionally, we observed the appearance of a slowly migrating, phosphorylated form of Bcl-2 connected with G2/M arrest of the cell cycle and apoptosis [37]. In summary, the analyses performed confirmed that compound **40** can activate apoptotic machinery in a dose-dependent manner.

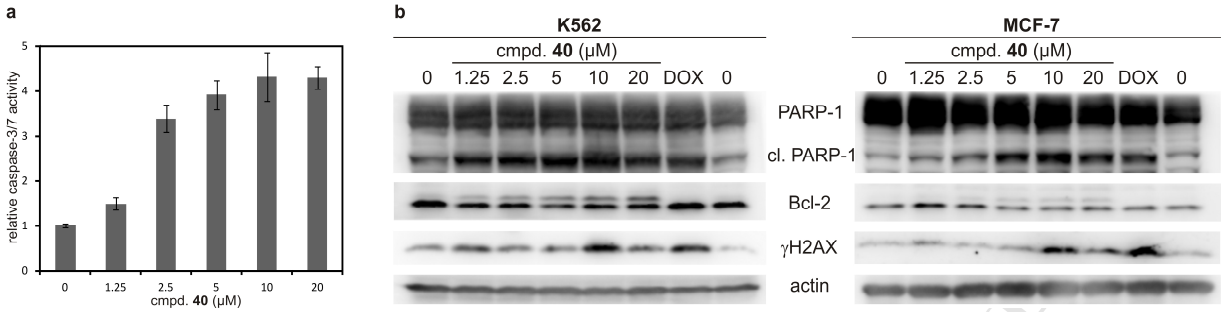


Fig. 3. Induction of apoptosis in cells treated with different doses of compound **40** for 24 h. (a) Caspase-3/7 activity was measured in lysates prepared from treated K562 cells using the fluorogenic substrate Ac-DEVD-AMC and normalized against untreated control cell lysates. (b) Immunoblotting analysis of apoptosis-related proteins in treated K562 and MCF-7 cells. Doxorubucin (DOX, 1 μM) was used as a control. Actin levels were detected to verify equal loading.

To describe the mechanism of antimitotic action of the prepared compounds, we first explored CDK1/cyclin B, a well-known mitotic regulator, as a possible target, especially due to the structural similarity to 2,3,4,6-substituted pyrazolo[4,3-*c*]pyridines which have been identified as kinase inhibitors [12, 13]. The synthesized compounds **35-52** were screened for their inhibitory activity against CDK1, but the assay did not reveal any inhibition (data not shown). Further profiling of compound **40** against other mitotic kinases, including DAPK3, CDK1, CHK1, NIM1, AURKA, NDR1, NEK2/4/6, PIM2, PLK2/3, TTK and WEE1, did not point to any of these kinases as a target of compound **40** (data not shown). This finding was expected given that these compounds lack an H-bond donor-acceptor motif which is common in most kinase inhibitors, including the previously mentioned pyrazolo[4,3-*c*]pyridines [12, 13].

3. Conclusion

We detail the development of an efficient approach for the synthesis of variously substituted 2*H*-pyrazolo[4,3-*c*]pyridines, employing Sonogashira cross-coupling and a subsequent substituent-tolerant annulation reaction in the presence of ammonia. As a result of this, a library of 2*H*-pyrazolo[4,3-*c*]pyridines, varying the substituents at the 2-, 4-, and 6-positions, was synthesized. These compounds were also evaluated for their cytotoxicity against K562 and MCF-7 cancer cell

lines. The tested compounds exhibited anticancer activity *in vitro* through arresting cell cycle in mitosis and induction of apoptosis, although, the mechanism of cellular action remains unclear.

4. Experimental Section

4.1. Chemistry

All starting materials were obtained from commercial suppliers and used without further purification. Melting points were determined on a Büchi M-565 melting point apparatus and were uncorrected. Mass spectra were obtained on a Shimadzu LCMS 2020 Single Quadrupole Liquid Chromatograph Mass Spectrometer. IR spectra in KBr pellets were recorded on a Bruker Tensor 27 spectrometer and are reported in wave numbers (cm^{-1}). High-resolution ESI-TOF mass spectra were measured on a Bruker maXis spectrometer. ^{1}H NMR, ^{13}C NMR and ^{15}N NMR spectra were recorded from $CDCl_3$ or DMSO-d_6 solutions at 25 °C on a Bruker Avance III 700 instrument (700 MHz for ^{1}H, 176 MHz for ^{13}C) equipped with a 5 mm TCI ^{1}H-^{13}C/^{15}N/D *z*-gradient cryoprobe. The solvent (residual) signals were used as internal standards and were related to TMS, with δ 7.26 ppm (^{1}H) and δ 77.00 ppm (^{13}C) for $CDCl_3$ and with 2.50 ppm (^{1}H) and δ 39.52 ppm (^{13}C) for DMSO-d_6. ^{15}N NMR spectra were referenced against neat, external nitromethane. The full and unambiguous assignments of ^{1}H, ^{13}C and ^{15}N NMR resonances were achieved using combined applications of standard NMR spectroscopic techniques such as APT, COSY, TOCSY, NOESY, gs-HSQC and gs-HMBC. For chromatographic separations, silica gel 60 (230–400 mesh, Merck) was used.

4.1.1. 3-(Benzyloxy)-1-methyl-1*H*-pyrazole (3) [27, 28]. A solution of 3-hydroxy-1-methyl-1*H*-pyrazole (**1**) [38] (710 mg, 7.2 mmol) in dry DMF (20 mL) was cooled to 0 ºC under inert atmosphere and NaH (60% dispersion in mineral oil, 290 mg, 7.2 mmol) was added portion wise. After stirring mixture for 15 min benzyl chloride (0.82 mL, 7.2 mmol) was added drop wise. The mixture was stirred at 60 ºC for 1 hour, then poured into water and extracted with ethyl acetate. The organic layers were combined, washed with brine, dried over Na_2SO_4, filtrated, and the solvent was evaporated. The residue was purified by column chromatography (SiO_2, eluent: ethyl acetate/*n*-hexane, 1:7, v/v) to give pure **3** as a brown liquid. Yield 90%, 1205 mg. IR (ν_{max}, cm^{-1}): 3118, 3089, 3064, 3032, 3008 (CH_{arom}), 2931 (CH_{aliph}), 1537, 1491, 1429, 1360, 1223, 1052, 1018 (C=C, C–N), 731, 696, 658, 457 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.75 (s, 3H, CH_3), 5.20 (s, 2H, CH_2Ph), 5.66 (d, ^{3}J(4-H,5-H) = 2.4 Hz, 1H, 4-

H), 7.13 (d, ^{3}J(5-H,4-H) = 2.4 Hz, 1H, 5-H), 7.30-7.33 (m, 1H, Ph 4-H), 7.36-7.39 (m, 2H, Ph 3,5-H), 7.45-7.48 (m, 2H, Ph 2,6-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 39.0 (CH_3), 70.9 (CH_2Ph), 90.5 (C-4), 127.8 (Ph C-2,6), 128.0 (Ph C-4), 128.5 (Ph C-3.5), 131.4 (C-5), 137.3 (Ph C-1), 163.3 (C-3). MS m/z (%): 189 ($[M+H]^+$, 100). HRMS (ESI) for $C_{11}H_{12}N_2ONa$ ($[M+Na]^+$) calcd 211.0842, found 211.0842.

4.1.2. 3-(Benzyloxy)-1-methyl-1*H*-pyrazole-4-carbaldehyde (5). Phosphorus oxychloride (1.59 mL, 17 mmol) was added dropwise to DMF (1.32 mL, 17 mmol) at –10 °C. Then **3** (800 mg, 4.25 mmol) was added to the Vilsmeier-Haack complex and the reaction mixture was heated at 70 °C for 12 hours. After neutralization with 10% aq $NaHCO_3$, the preticipate was filtered off and recrystallized from DCM to give pure **5** as a white solid. Yield 85%, 775 mg, mp 54-56 °C. IR (ν_{max}, cm^{-1}): 3121, 3099, 3045 (CH_{arom}), 2992, 2947 (CH_{aliph}), 1666 (C=O), 1588, 1577, 1541, 1510, 1315, 1181 (C=C, C–N), 894, 883, 706 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.77 (s, 3H, CH_3), 5.32 (s, 2H, CH_2Ph), 7.32-7.36 (m, 1H, Ph 4-H), 7.36-7.40 (m, 2H, Ph 3,5-H), 7.46-7.47 (m, 2H, Ph 2,6-H), 7.69 (s, 1H, 5-H), 9.76 (s, 1H, CHO). ^{13}C NMR (176 MHz, $CDCl_3$): δ 39.8 (CH_3), 71.0 (CH_2Ph), 109.6 (C-4), 128.0 (Ph C-2,6), 128.3 (Ph C-4), 128.6 (Ph C-3.5), 133.4 (C-5), 136.4 (Ph C-1), 163.3 (C-3), 183.1 (CHO). MS m/z (%): 217 ($[M+H]^+$, 100). HRMS (ESI) for $C_{11}H_{12}N_2ONa$ ($[M+Na]^+$) calcd 239.0791, found 239.0791.

4.1.3. 3-Hydroxy-1-methyl-1*H*-pyrazole-4-carbaldehyde (7). To a solution of **5** (570 mg, 2.6 mmol) in toluene (10 mL) TFA (10 mL) was added. The mixture was stirred at room temperature for 48 hours. Toluene and TFA were evaporated. The residue was purified by column chromatography (SiO_2, eluent: ethyl acetate/*n*-hexane, 1:1, v/v) to give pure **7** as a colorless solid. Yield 90%, 295 mg, mp 201-202 °C. IR (ν_{max}, cm^{-1}): 3291 (OH), 3099 (CH_{arom}), 2992, 2947 (CH_{aliph}), 1666 (C=O), 1541, 1510, 1315, 1181 (C=C, C–N). ^{1}H NMR (700 MHz, DMSO-d_6): δ 3.67 (s, 3H, CH_3), 8.04 (s, 1H, 5-H), 9.60 (s, 1H, CHO), 10.90 (s, 1H, OH). ^{13}C NMR (176 MHz, DMSO-d_6): δ 38.9 (CH_3), 108.4 (C-4), 134.0 (C-5), 161.9 (C-3), 182.6 (CHO). MS m/z (%): 127 ($[M+H]^+$, 100). HRMS (ESI) for $C_5H_6N_2O_2Na$ ($[M+Na]^+$) calcd 149.0322, found 149.0321.

4.1.4. 4-Formyl-1-methyl-1*H*-pyrazol-3-yl trifluoromethanesulfonate (9). Pyrazole **7** (250 mg, 2 mmol), trifluormethansulfonic anhydride (1 mL, 6 mmol) and TEA (1 mL, 7.2 mmol) were dissolved in DCM (20 mL) and the mixture was stirred at room temperature for 1 hour. The reaction mixture was poured into water and extracted with ethyl acetate. Combined organic

layers were washed with brine and dried over Na_2SO_4, the solvent was evaporated. The residue was purified by flash chromatography (SiO_2, eluent: ethyl acetate/*n*-hexane, 1:4, v/v) to give **9** as a brown solid. Yield 85%, 435 mg, mp 54-55 °C. IR (ν_{max}, cm^{-1}): 3091, 3043, 3017 (CH_{arom}), 2958, 2918 (CH_{aliph}), 1675 (C=O), 1552, 1429, 1205, 1135, 884, 875, 794, 744, 601, 511 (C-C, C-N, C-F). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.93 (s, 3H, CH_3), 7.87 (s, 1H, 5-H), 9.80 (s, 1H, CHO). ^{13}C NMR (176 MHz, $CDCl_3$): δ 40.7 (CH_3), 113.6 (C-4), 116.0, 117.8, 119.6, 121.5 (CF_3 ^1J = 321.2 Hz), 134.7 (C-5), 151.2 (C-3), 181.0 (CHO). MS m/z (%): 259 ($[M+H]^+$, 100). HRMS (ESI) for $C_6H_5F_3N_2O_4SNa$ ($[M+Na]^+$) calcd 280.9814, found 280.9814.

4.1.5. 1-Phenyl-1*H*-pyrazol-3-yl isobutyrate (12). To a solution of 3-hydroxy-1-phenyl-1*H*-pyrazole (**2**) [33] (470 mg, 2.9 mmol) in DCM (10 mL) pyridine (2.3 mL) and isobutyrylchloride (0.33 mL, 3.2 mmol) were added. The mixture was stirred at room temperature for 30 min then poured into water and extracted with DCM. The organic layers were combined, washed with brine, dried over Na_2SO_4, filtrated, the solvent was evaporated. The residue was purified by column chromatography (SiO_2, eluent: ethyl acetate/*n*-hexane, 1:4, v/v) to give pure **12** as a brown liquid. Yield 90%, 601 mg. IR (ν_{max}, cm^{-1}): 3134, 3064, 3050 (CH_{arom}), 2977, 2938, 2912 (CH_{aliph}), 1761 (C=O), 1599, 1532, 1453, 1391, 1124, 1113, 1092 (C=C, C–N), 752, 688 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 1.36 (d, J = 7.1 Hz, 6H, $CH(CH_3)_2$), 2.85-2.90 (m, 1H, $CH(CH_3)_2$), 6.41 (d, ^3J(4-H,5-H) = 2.6 Hz, 1H, 4-H), 7.28-7.30 (m, 1H, Ph 4-H), 7.44-7.46 (m, 2H, Ph 3,5-H), 7.64-7.66 (m, 2H, Ph 2,6-H), 7.86 (d, ^3J(5-H,4-H) = 2.6 Hz, 1H, 5-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 18.8 ($CH(CH_3)_2$), 34.1 ($CH(CH_3)_2$), 98.8 (C-4), 118.7 (Ph C-2,6), 126.5 (C-5), 127.7 (Ph C-4), 129.4 (Ph C-3,5), 139.7 (Ph C-1), 156.8 (C-3), 174.2 (O-C=O). MS m/z (%): 231 ($[M+H]^+$, 100). HRMS (ESI) for $C_{13}H_{14}N_2O_2Na$ ($[M+Na]^+$) calcd 253.0947, found 253.0947.

4.1.6. 1-(3-Hydroxy-1-phenyl-1*H*-pyrazol-4-yl)-2-methylpropan-1-one (14). To a solution of $AlCl_3$ (2.453 g, 18.4 mmol) in CS_2 (8 mL) a solution of **12** (350 mg, 1.5 mmol) in CS_2 (28 mL) was added drop wise at 0 °C. The mixture was stirred at 55 °C for 3 hours. After neutralization with ice-cold water (33 mL) and 6N HCl (15 mL), the preticipate was filtered off and purified by flash chromatography (SiO_2, eluent: ethyl acetate/n-hexane, 1:4, v/v) to give pure **10** as a white solid. Yield 89%, 310 mg, mp 97-98 °C. IR (ν_{max}, cm^{-1}): 3309 (OH), 3122, 3068, 3052 (CH_{arom}), 2965, 2932 (CH_{aliph}), 1656 (C=O), 1559, 1530, 1508, 1459, 1319, 1233, 1217 (C=C, C–N), 747, 685, 671 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 1.07 (d, J = 6.9

Hz, 6H, CH(C*H*$_3$)$_2$), 3.33-3.36 (m, 1H, C*H*(CH$_3$)$_2$), 7.29-7.31 (m, 1H, Ph 4-H), 7.47-7.49 (m, 2H, Ph 3,5-H), 7.80-7.81 (m, 2H, Ph 2,6-H), 8.87 (s, 1H, 5-H), 11.12 (s, 1H, OH). ^{13}C NMR (176 MHz, $CDCl_3$): δ 18.7 (CH(*C*H$_3$)$_2$), 36.9 (*C*H(CH$_3$)$_2$), 109.4 (C-4), 118.1 (Ph C-2,6), 126.5 (Ph C-4), 129.6 (Ph C-3,5), 131.4 (C-5), 138.9 (Ph C-1), 161.4 (C-3), 198.6 (C=O). MS m/z (%): 231 ([M+H]$^+$, 100). HRMS (ESI) for $C_{13}H_{14}N_2O_2Na$ ([M+Na]$^+$) calcd 253.0947, found 253.0947.

4.1.7. 4-Isobutyryl-1-phenyl-1*H*-pyrazol-3-yltrifluoromethanesulfonate (16). This compound was synthesized in analogy to compound **9** from pyrazole **14** (252 mg, 2 mmol). **16** was obtained as a white solid. Yield 96%, 495 mg, mp 96-97 °C. IR (ν_{max}, cm^{-1}): 3094, 3061 (CH$_{arom}$), 2980, 2937, 2918 (CH$_{aliph}$), 1555, 1452, 1427, 1231, 1213, 1201, 1138 (C=C, C–N, C-F), 973, 881, 760, 740, 607, 599, 505 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 1.23-1.24 (m, 6H, CH(C*H*$_3$)$_2$), 3.15-3.18 (m, 1H, C*H*(CH$_3$)$_2$), 7.39-7.41 (m, 1H, Ph 4-H), 7.49-7.51 (m, 2H, Ph 3,5-H), 7.66-7.67 (m, 2H, Ph 2,6-H), 8.35 (s, 1H, 5-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 18.7 (CH(*C*H$_3$)$_2$), 38.8 (*C*H(CH$_3$)$_2$), 113.6 (C-4), 116.0, 117.8, 119.5 (Ph C-2,6), 119.6, 121.5 (CF$_3$ ^1J = 322.1 Hz), 128.5 (Ph C-4), 129.9 (Ph C-3,5), 130.8 (C-5), 138.5 (Ph C-1), 151.9 (C-3), 196.6 (C=O). MS m/z (%): 363 ([M+H]$^+$, 100). HRMS (ESI) for $C_6H_5F_3N_2O_4SNa$ ([M+Na]$^+$) calcd 385.0440, found 385.0440.

4.1.8. General procedure for the preparation of 4-alkynyl-1-phenyl-1*H*-pyrazole-4-carbaldehydes, ethanones and propanones by Sonogashira cross-coupling reaction.

To a solution of appropriate pyrazole **9**, **10**, **15** or **16** (0.5 mmol) in dry DMF (1 mL) under argon atmosphere TEA (4.0 mL, 2.5 mmol), appropriate acetylene (0.75 mmol), Pd(PPh$_3$)$_2$Cl$_2$ (35 mg, 0.05 mmol) and CuI (18 mg, 0.1 mmol) were added. The mixture was stirred for the given time under argon atmosphere at 70 °C, diluted with water, and the extraction was done with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4, and the solvent was evaporated. The residue was purified by flash chromatography (SiO_2, eluent: ethyl acetate/n-hexane, 1:8, v/v) to yield compounds **17-34**.

4.1.8.1. 1-Methyl-3-(phenylethynyl)-1*H*-pyrazole-4-carbaldehyde (**17**). The reaction mixture was stirred for 8 hours. Brown amorphous solid, yield 75%, 105 mg, mp 123-124 °C. IR (ν_{max}, cm^{-1}): 3100, 3057, 3042 (CH$_{arom}$), 2954, 2925 (CH$_{aliph}$), 2221 (C≡C), 1681 (CHO), 1598, 1560, 1533, 1497, 1441, 1181 (C=C, C–N), 909, 880, 775, 755, 690 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.85 (s, 3H, CH_3), 7.24-7.7(m, 3H, Ph 3,4,5-H), 7.46-7.47 (m, 2H, Ph 2,6-H), 7.90 (s, 1H, 5-H), 9.88 (s, 1H, CHO). ^{13}C NMR (176 MHz, $CDCl_3$): δ

40.1 (CH_3), 79.0 (*C*≡C-Ph), 94.1 (C≡*C*-Ph), 113.6 (C-3), 122.0 (Ph C-1), 125.0 (C-4), 128.6 (Ph C-2,6), 134.8 (C-5), 184.3 (CHO). MS m/z (%): 211 ($[M+H]^+$, 100). HRMS (ESI) for $C_{16}H_{10}N_2OSNa$ ($[M+Na]^+$) calcd 233.0685, found 233.0685.

4.1.8.2. 1-Phenyl-3-(thiophen-3-ylethynyl)-1*H*-pyrazole-4-carbaldehyde (19). The reaction mixture was stirred for 8 hours. White solid, yield 75%, 105 mg, mp 123-124 °C. IR (ν_{max}, cm^{-1}): 3126, 3036 (CH_{arom}), 2834, 2788 (CH_{aliph}), 2229 (C≡C), 1676 (C=O), 1596, 1561, 1462, 1409, 1297, 1125 (C=C, C–N), 909, 873, 815, 621, 598, 512 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 7.28 (d, ^3J(4-H,5-H) = 4.9 Hz, 1H, Th 4-H), 7.34 (dd, ^3J(5-H,4-H) = 4.9 Hz, ^4J(5-H,2-H) = 2.9 Hz, 1H, Th 5-H), 7.40-7.42 (m, 1H, Ph 4-H), 7.68 (d, ^4J(2-H,5-H) = 2.9 Hz, 1H, Th 2-H), 7.50-7.52 (m, 2H, Ph 3,5-H), 7.74-7.76 (m, 2H, Ph 2,6-H), 8.44 (s, 1H, 5-H), 10.09 (s, 1H, CHO). ^{13}C NMR (176 MHz, $CDCl_3$): δ 78.6 (*C*≡C-Th), 90.3 (C≡*C*-Th), 120.0 (Ph C-2,6), 120.9 (Th C-3), 125.91 (C-4), 125.95 (Th C-5), 128.5 (Ph C-4), 128.7 (C-5), 129.9 (Ph C-3,5), 130.0 (Th C-4), 130.8 (Th C-2), 138.7 (C-3), 138.9 (Ph C-1), 182.7 (CHO). ^{15}N NMR (71 MHz, $CDCl_3$): δ −158.2 (N-1), −72.0 (N-2). MS m/z (%): 279 ($[M+H]^+$, 100). HRMS (ESI) for $C_{16}H_{10}N_2OSNa$ ($[M+Na]^+$) calcd 301.0406, found 301.0406.

4.1.8.3. 3-(Cyclopropylethynyl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde (20). The reaction mixture was stirred for 1 hour. White solid, yield 86%, 103 mg, mp 90-91 °C. IR (ν_{max}, cm^{-1}): 3121, 3065, 3014 (CH_{arom}), 2881, 2782 (CH_{aliph}), 2232 (C≡C), 1677 (C=O), 1599, 1530, 1504, 1361, 1226 (C=C, C–N), 865, 849, 801, 757, 704, 685, 506 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.92-0.98 (m, 4H, $CH(CH_2)_2$, 1.52-1.57 (m, 1H, $CH(CH_2)_2$), 7.37-7.41 (m, 1H, Ph 4-H), 7.47-7.52 (m, 2H, Ph 3,5-H), 7.70-7.73 (m, 2H, Ph 2,6-H), 8.37 (s, 1H, 5-H), 9.97 (s, 1H, CHO). ^{13}C NMR (176 MHz, $CDCl_3$): δ 0.4 ($CH(CH_2)_2$), 9.1 ($CH(CH_2)_2$), 65.5 (*C*≡C($CH(CH_2)_2$), 100.2 (C≡*C*($CH(CH_2)_2$), 119.9 (Ph C-2,6), 125.9 (C-4), 128.3 (Ph C-4), 128.4 (C-5), 129.8 (Ph C-3,5), 138.9 (C-3), 139.3 (Ph C-1), 184.8 (CHO). ^{15}N NMR (71 MHz, $CDCl_3$): δ −157.6 (N-1), N-2 was not found. MS m/z (%): 237 ($[M+H]^+$, 100). HRMS (ESI) for $C_{15}H_{12}N_2ONa$ ($[M+Na]^+$) calcd 359.0842, found 359.0844.

4.1.8.4. 3-(Hept-1-yn-1-yl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde (22). The reaction mixture was stirred for 4 hours. Brown liquid, yield 92%, 128 mg. IR (ν_{max}, cm^{-1}): 3124, 3053 (CH_{arom}), 2956, 2931, 2860 (CH_{aliph}), 2243 (C≡C), 1684 (C=O), 1599, 1531, 1504, 1464, 1398, 1296 (C=C, C–N), 956, 757, 689, 509 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.89-0.93 (m, 3H, $C_4H_8CH_3$), 1.34-1.39 (m, 2H, $C_3H_6CH_2CH_3$), 1.44-1.48 (m, 2H,

$C_2H_4CH_2C_2H_5$), 1.65-1.69 (m, 2H, $CH_2CH_2C_3H_7$), 2.48-2.51 (m, 2H, $CH_2C_4H_9$), 7.36-7.39 (m, 1H, Ph 4-H), 7.46-7.49 (m, 2H, Ph 3,5-H), 7.69-7.72 (m, 2H, Ph 2,6-H), 8.38 (s, 1H, 5-H), 9.99 (s, 1H, CHO). ^{13}C NMR (176 MHz, $CDCl_3$): δ 14.0 ($C_4H_8CH_3$), 19.5 ($CH_2C_4H_9$), 22.2 ($C_3H_6CH_2CH_3$), 28.0 ($CH_2CH_2C_3H_7$), 31.1 ($C_2H_4CH_2C_2H_5$), 70.3 ($C_5H_{11}C{\equiv}C$) 97.2 ($C_5H_{11}C{\equiv}C$), 119.8 (Ph C-2,6), 125.7 (C-4), 128.2 (Ph C-4), 128.3 (C-5), 129.7 (Ph C-3,5), 138.8 (Ph C-1), 139.3 (C-3), 184.8 (CHO). ^{15}N NMR (71 MHz, $CDCl_3$): δ –158.6 (N-1), –72.4 (N-2). MS m/z (%): 267 ($[M+H]^+$, 100). HRMS (ESI) for $C_{17}H_{18}N_2ONa$ ($[M+H]^+$) calcd 289.1306, found 289.1311.

4.1.8.5. 3-(4-Hydroxybut-1-yn-1-yl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde (23). The reaction mixture was stirred for 4 hours. White solid, yield 90%, 108 mg, mp 111-112 °C. IR (ν_{max}, cm^{-1}): 3454 (OH), 3126, 30965 (CH_{arom}), 2929 (CH_{aliph}), 2240 (C≡C), 1679 (C=O), 1598, 1531, 1504, 1462, 1402, 1362, 1315, 1226, 1051 (C=C, C–N), 866, 812, 796, 755, 685 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 2.74-2.76 (m, 2H, $C{\equiv}CCH_2CH_2OH$), 3.87-3.88 (m, 2H, $C{\equiv}CCH_2CH_2OH$), 7.35-7.37 (m, 1H, Ph 4-H), 7.44-7.47 (m, 2H, Ph 3,5-H), 7.66-7.68 (m, 2H, Ph 2,6-H), 8.37 (s, 1H, 5-H), 9.95 (CHO). ^{13}C NMR (176 MHz, $CDCl_3$): δ 24.1($C{\equiv}CCH_2CH_2OH$), 60.8 ($C{\equiv}CCH_2CH_2OH$), 72.4 ($C{\equiv}CCH_2CH_2OH$), 94.0 ($C{\equiv}CCH_2CH_2OH$), 119.8 (NPh C-2,6), 125.8 (C-4), 128.4 (Ph C-4), 129.5 (Ph C-3,5), 129.8 (C-5), 138.1 (C-3), 138.7 (Ph C-1), 184.8 (*C*HO). MS m/z (%): 241 ($[M+H]^+$, 100), 263 ($[M+Na]^+$, 30). HRMS (ESI) for $C_{14}H_{12}N_2ONa$ ($[M+Na]^+$) calcd 263.0792, found 263.0791.

4.1.8.6. 1-Phenyl-3-((trimethylsilyl)ethynyl)-1*H*-pyrazole-4-carbaldehyde (24). The reaction mixture was stirred for 10 hours. White solid, yield 78%, 105 mg, mp 97-98 °C. IR (ν_{max}, cm^{-1}): 3128, 3067 (CH_{arom}), 2960, 2854 (CH_{aliph}), 2167 (C≡C), 1678 (C=O), 1598, 1531, 1503, 1363, 1251, 1223 (C=C, C–N), 956, 838, 751, 726, 683 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.30 (s, 9H, TMS), 7.37-7.41 (m, 1H, Ph 4-H), 7.47-7.51 (m, 2H, Ph 3,5-H), 7.70-7.72 (m, 2H, Ph 2,6-H), 8.39 (s, 1H, 5-H), 10.01 (s, 1H, CHO). ^{13}C NMR (176 MHz, $CDCl_3$): δ 0.2 (TMS), 93.6 (TMS*C*≡C), 102.0 (TMSC≡*C*), 120.1 (Ph C-2,6), 126.2 (C-4), 128.3 (C-5), 128.5 (Ph C-4), 129.9 (Ph C-3,5), 138.7 (C-3), 138.8 (Ph C-1), 184.7 (CHO). ^{15}N NMR (71 MHz, $CDCl_3$): δ –157 (N-1), N-2 was not found. MS m/z (%): 269 ($[M+H]^+$, 100). HRMS (ESI) for $C_{15}H_{16}N_2OSiNa$ ($[M+Na]^+$) calcd 291.0924, found 291.0928.

4.1.8.7. 1-(1-Phenyl-3-(thiophen-3-ylethynyl)-1*H*-pyrazol-4-yl)ethanone (26). The reaction mixture was stirred for 8 hours. White solid, yield 66%, 97 mg, mp 116-117 °C. IR (ν_{max}, cm^{-1}):

3117, 3054, 3022 (CH_{arom}), 2996, 2955, 2917 (CH_{aliph}), 2232 (C≡C), 1655 (C=O), 1597, 1514, 1508, 1351, 1264, 1232, 1058 (C=C, C–N), 872, 774, 761, 708, 691, 680, 620, 503 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 2.73 (s, 3H, CH_3), 7.28 (d, ^3J(4-H,5-H) = 5.2 Hz, 1H, Th 4-H), 7.35-7.37 (m, 1H, Th 5-H), 7.39-7.42 (m, 1H, Ph 4-H), 7.50-7.52 (m, 2H, Ph 3,5-H), 7.68 (d, ^4J(2-H,5-H) = 2.9 Hz, 1H, Th 2-H), 7.75-7.77 (m, 2H, Ph 2,6-H), 8.47 (s, 1H, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.1 (CH_3), 80.8 (ThC≡*C*), 90.1 (Th*C*≡C), 119.7 (Ph C-2,6), 121.1 (Th C-3), 125.8 (Th C-5), 126.9 (C-4), 128.1 (Ph C-4), 129.7 (Th C-4), 129.7 (Ph C-3,5), 129.9 (C-5), 130.2 (Th C-2), 136.2 (C-3), 138.8 (Ph C-1), 192.2 (CO). ^{15}N NMR (71 MHz, $CDCl_3$): δ −160.4 (N-1), −72.5 (N-2). MS m/z (%): 293 ($[M+H]^+$, 100). HRMS (ESI) for $C_{17}H_{12}N_2OSiNa$ ($[M+Na]^+$) calcd 315.0563, found 315.0561.

4.1.8.8. 1-(3-(Cyclopropylethynyl)-1-phenyl-1*H*-pyrazol-4-yl)ethanone (27). The reaction mixture was stirred for 1 hour. White solid, yield 80%, 100 mg, mp 127-128 °C. IR (ν_{max}, cm^{-1}): 3129, 3078 (CH_{arom}), 2993, 2956, 2923 (CH_{aliph}), 2233 (C≡C), 1670 (C=O), 1599, 1521, 1448, 1362, 1260, 1241, 1221 (C=C, C–N), 977, 940, 863, 751, 705, 683 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.90-0.96 (m, 4H, CH(CH_2)$_2$), 1.53-1.57 (m, 1H, C*H*(CH_2)$_2$), 2.62 (s, 3H, COC*H*$_3$), 7.34-7.36 (m, 1H, Ph 4-H), 7.45-7.47 (m, 2H, Ph 3,5-H), 7.69 (m, 2H, Ph 2,6-H), 8.38 (s, 1H, 5-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 0.4 (*C*H(CH_2)$_2$), 8.8 (CH(*C*H_2)$_2$), 29.1 (CO*C*H_3), 67.8 ((CH_2)$_2$CHC≡*C*), 99.9 ((CH_2)$_2$CH*C*≡C), 119.8 (Ph C-2,6), 126.9 (C-4), 128.0 (Ph C-4), 129.7 (C-5), 129.8 (Ph C-3,5), 136.7 (C-3), 139.0 (Ph C-1), 192.6 (C=O). ^{15}N NMR (71 MHz, $CDCl_3$): δ –160.2 (N-1), N-2 was not found. MS m/z (%): 251 ($[M+H]^+$, 100). HRMS (ESI) for $C_{16}H_{14}N_2O$ ($[M+H]^+$) calcd 273.0998, found 273.0998.

4.1.8.9. 1-(3-(Hex-1-yn-1-yl)-1-phenyl-1*H*-pyrazol-4-yl)ethanone (28). The reaction mixture was stirred for 1 hour. Yellow liquid, yield 75%, 106 mg. IR (ν_{max}, cm^{-1}): 3130, 3064 (CH_{arom}), 2957, 2928, 2860 (CH_{aliph}), 2240 (C≡C), 1666 (C=O), 1522, 1457, 1362, 1262, 1245, 1217 (C=C, C–N), 751, 706, 685 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.94 (t, *J* = 7.4 Hz, 3H, $C_3H_6CH_3$), 1.48-1.51 (m, 2H, $C_2H_4CH_2CH_3$), 1.62-1.66 (m, 2H, $CH_2CH_2C_2H_5$), 2.49-2.51 (m, 2H, $CH_2C_3H_7$), 2.51 (s, 1H, COC*H*$_3$), 7.32-7.34 (m, 1H, Ph 4-H), 7.43-7.46 (m, 2H, Ph 3,5-H), 7.69-7.70 (m, 2H, Ph 2,6-H), 8.38 (s, 1H, 5-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 13.7 ($C_3H_6CH_3$), 19.4 ($CH_2C_3H_7$), 22.2 ($C_2H_4CH_2CH_3$), 29.0 (CH_3), 30.3 ($CH_2CH_2C_2H_5$), 72.7 (C_4H_9C≡*C*), 96.9 (C_4H_9C≡C), 119.7 (Ph C-2,6), 126.7 (C-4), 128.0 (Ph C-4), 129.7 (C-5), 129.8 (Ph C-3,5), 136.7 (C-3), 138.9 (Ph C-1), 192.5 (C=O). ^{15}N NMR (71

MHz, $CDCl_3$): δ –160.1 (N-1), –70.8 (N-2). MS m/z (%): 267 ([M+H]+, 100). HRMS (ESI) for $C_{17}H_{18}N_2ONa$ ($[M+Na]^+$) calcd 289.1311, found 289.1312.

4.1.8.10. 1-(3-(Hept-1-yn-1-yl)-1-phenyl-1*H*-pyrazol-4-yl)ethanone (29). The reaction mixture was stirred for 4 hours. Brown liquid, yield 65%, 91 mg. IR (ν_{max}, cm^{-1}): 3130, 3064 (CH_{arom}), 2957, 2928, 2860 (CH_{aliph}), 2240 (C≡C), 1666 (C=O), 1522, 1457, 1362, 1262, 1245, 1217 (C=C, C–N), 751, 706, 685 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.91 (t, *J* = 7.3 Hz, 3H, $C_4H_8CH_3$), 1.33-1.38 (m, 2H, $C_3H_6CH_2CH_3$), 1.43-1.47 (m, 2H, $C_2H_4CH_2C_2H_5$), 1.64-1.69 (m, 2H, $CH_2CH_2C_3H_7$), 2.50 (t, *J* = 7.2 Hz, 2H, $CH_2C_4H_9$), 2.63 (CH_3), 7.31-7.36 (m, 1H, Ph 4-H), 7.44-7.46 (m, 2H, Ph 3,5-H), 7.69-7.70 (m, 2H, Ph 2,6-H), 8.39 (s, 1H, 5-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 14.0 ($C_4H_8CH_3$), 19.7 ($CH_2C_4H_9$), 22.3 ($C_3H_6CH_2CH_3$), 28.0 ($CH_2CH_2C_3H_7$), 29.0 (CH_3), 31.3 ($C_2H_4CH_2C_2H_5$), 72.7 ($C_5H_{11}C{\equiv}C$), 97.0 ($C_5H_{11}C{\equiv}C$), 119.7 (Ph C-2,6), 126.7 (C-4), 128.0 (Ph C-4), 128.6 (C-5), 129.7 (Ph C-3,5), 136.7 (C-3), 138.9 (Ph C-1), 192.5 (C=O). ^{15}N NMR (71 MHz, $CDCl_3$): δ –161.8 (N-1), N-2 was not found. MS m/z (%): 281 ($[M+H]^+$, 100). HRMS (ESI) for $C_{18}H_{20}N_2ONa$ ($[M+Na]^+$) calcd 303.1468, found 303.1468.

4.1.8.11. 1-(3-(4-Hydroxybut-1-yn-1-yl)-1-phenyl-1*H*-pyrazol-4-yl)ethanone (30). The reaction mixture was stirred for 4 hours. White solid, yield 86%, 109 mg, mp 121-122 °C. IR (ν_{max}, cm^{-1}): 3130, 3068, 3026 (CH_{arom}), 2995, 2961, 2945 (CH_{aliph}), 2240 (C≡C), 1666 (C=O), 1521, 1460, 1350, 1262, 1249, 1217 (C=C, C–N), 755, 731, 685, 577, 468 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 2.59 (CH_3), 2.75-2.77 (m, 2H, $C{\equiv}CCH_2CH_2OH$), 3.89-3.91 (m, 2H, $C{\equiv}CCH_2CH_2OH$), 7.36-7.38 (m, 1H, Ph 4-H), 7.47-7.49 (m, 2H, Ph 3,5-H), 7.69-7.71 (m, 2H, Ph 2,6-H), 8.36 (s, 1H, 5-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 24.3($C{\equiv}CCH_2CH_2OH$), 28.8($COCH_3$), 60.8 ($C{\equiv}CCH_2CH_2OH$), 74.7 ($C{\equiv}CCH_2CH_2OH$), 93.7 ($C{\equiv}CCH_2CH_2OH$), 119.7 (NPh C-2,6), 126.4 (C-4), 128.2 (Ph C-4), 129.8 (Ph C-3,5), 130.1 (C-5), 136.3 (C-3), 138.9 (Ph C-1), 192.0 ($COCH_3$). ^{15}N NMR (71 MHz, $CDCl_3$): δ –160.4 (N-1), –70.5 (N-2). MS m/z (%): 255 ($[M+H]^+$, 100). HRMS (ESI) for $C_{15}H_{14}N_2ONa$ ($[M+Na]^+$) calcd 277.0947, found 277.0947.

4.1.8.12. 1-(1-Phenyl-3-((trimethylsilyl)ethynyl)-1*H*-pyrazol-4-yl)ethanone (31). The reaction mixture was stirred for 12 hours. White solid, yield 83%, 117 mg, mp 89-90 °C. IR (ν_{max}, cm^{-1}): 3129, 3065 (CH_{arom}), 2959, 2899 (CH_{aliph}), 2167 (C≡C), 1666 (C=O), 1523, 1507, 1442, 1363, 1261, 1206 (C=C, C–N), 858, 847, 755, 690 (CH=CH of monosubstituted benzene). ^{1}H NMR

(700 MHz, $CDCl_3$): δ 0.30 (s, 9H, TMS), 2.66 (s, 3H, CH_3), 7.36-7.38 (m, 1H, Ph 4-H), 7.46-7.48 (m, 2H, Ph 3,5-H), 7.70-7.71 (m, 2H, Ph 2,6-H), 8.40 (s, 1H, 5-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 0.3 (TMS), 29.1 (CH_3), 96.3 (TMSC≡*C*), 101.9 (TMS*C*≡C), 119.9 (Ph C-2,6), 127.5 (C-4), 128.2 (C-5), 129.8 (Ph C-4), 129.9 (Ph C-3,5), 135.9 (C-3), 138.9 (Ph C-1), 192.5 (*C*OCH_3). ^{15}N NMR (71 MHz, $CDCl_3$): δ –158.7 (N-1), N-2 was not found. MS m/z (%): 283 ($[M+H]^+$, 100). HRMS (ESI) for $C_{16}H_{18}N_2OSiNa$ ($[M+Na]^+$) calcd 305.1081, found 305.1081.

4.1.8.13. 2-Methyl-1-(1-phenyl-3-(phenylethynyl)-1*H*-pyrazol-4-yl)propan-1-one (32). The reaction mixture was stirred for 1 hour. White solid, yield 90%, 141 mg, mp 105-106 °C. IR (ν_{max}, cm^{-1}): 3121, 3061 (CH_{arom}), 2968, 2869 (CH_{aliph}), 2265 (C≡C), 1664 (C=O), 1521, 1443, 1350, 1227 (C=C, C–N), 989, 875, 751, 685 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 1.29 (d, *J* = 6.9 Hz, 6H, $CH(CH_3)_2$), 3.72-3.76 (m, 1H, $CH(CH_3)_2$, 7.37-7.40 (m, 4H, NPh 4-H, CPh 3,4,5-H), 7.48-7.50 (m, 2H, NPh 3,5-H), 7.61-7.62 (m, 2H, CPh 2,6-H), 7.75-7.76 (m, 2H, NPh 2,6-H), 8.47 (s, 1H, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.9 ($CH(CH_3)_2$), 38.0 ($CH(CH_3)_2$), 81.3 (PhC≡*C*), 94.0 (Ph*C*≡C), 119.9 (NPh C-2,6), 122.2 (CPh C-1), 125.8 (C-4), 128.1 (NPh C-4), 128.6 (CPh C-3,5), 129.3 (CPh C-4), 129.8 (NPh C-3,5), 130.6 (C-5), 131.9 (CPh C-2,6), 135.7 (C-3), 139.0 (NPh C-1), 198.9 (C=O). ^{15}N NMR (71 MHz, $CDCl_3$): δ –158.6 (N-1), N-2 was not found. MS m/z (%): 315 ($[M+H]^+$, 100). HRMS (ESI) for $C_{21}H_{18}N_2ONa$ ($[M+Na]^+$) calcd 337.1311, found 337.1311.

4.1.8.14. 1-(3-(Hex-1-yn-1-yl)-1-phenyl-1*H*-pyrazol-4-yl)-2-methylpropan-1-one (33). The reaction mixture was stirred for 1 hour. Colorless liquid, yield 81%, 119 mg. IR (ν_{max}, cm^{-1}): 3122, 3056 (CH_{arom}), 2961, 2929, 2871, 2855 (CH_{aliph}), 2237 (C≡C), 1665 (C=O), 1519, 1436, 1352, 1228 (C=C, C–N), 959, 879, 857, 756, 686 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.93-0.95 (m, 3H, $C_3H_6CH_3$), 1.22 (d, *J* = 7.0 Hz, 6H, $CH(CH_3)_2$), 1.47-1.52 (m, 2H, $C_2H_4CH_2CH_3$), 1.62-1.66 (m, 2H, $CH_2CH_2C_2H_5$), 2.49-2.51 (m, 2H, $CH_2C_3H_7$), 3.64-3.70 (m, 1H, $CH(CH_3)_2$, 7.31-7.36 (m, 1H, NPh 4-H), 7.44-7.48 (m, 2H, NPh 3,5-H), 7.68-7.72 (m, 2H, NPh 2,6-H), 8.41 (s, 1H, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.7 ($C_3H_6CH_3$), 18.8 ($CH(CH_3)_2$), 19.4 ($CH_2C_3H_7$) 22.2 ($C_2H_4CH_2CH_3$), 30.4 ($CH_2CH_2C_2H_5$), 37.6 ($CH(CH_3)_2$), 72.6 (C_4H_9C≡*C*), 96.0 (C_4H_9*C*≡C), 119.7 (NPh C-2,6), 125.3 (C-4), 127.9 (NPh C-4), 129.6 (NPh C-3,5), 130.3 (C-5), 136.1 (C-3), 139.0 (NPh C-1), 199.2 (C=O). ^{15}N NMR (71 MHz, $CDCl_3$): δ –160.5 (N-1), –72.2 (N-2). MS m/z (%): 295 ($[M+H]^+$, 100). HRMS (ESI) for $C_{19}H_{24}N_2O_2Na$ ($[M+Na+H_2O]^+$) calcd 335.1730, found 335.1723.

4.1.8.15. 2-Methyl-1-(1-phenyl-3-((trimethylsilyl)ethynyl)-1*H*-pyrazol-4-yl)propan-1-one (34). The reaction mixture was stirred for 12 hours. White solid, yield 87%, 135 mg, mp 91-92 °C. IR (ν_{max}, cm^{-1}): 3124, 3060 (CH_{arom}), 2987, 2971, 2933, 2903 (CH_{aliph}), 2166 (C≡C), 1665 (C=O), 1518, 1435, 1354, 1247, 1226 (C=C, C–N), 873, 848, 757, 705 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.30 (s, 9H, TMS), 1.20-1.26 (m, 6H, $CH(CH_3)_2$), 3.72-3.76 (m, 1H, $CH(CH_3)_2$, 7.36-7.39 (m, 1H, Ph 4-H), 7.45-7.49 (m, 2H, Ph 3,5-H), 7.71-7.72 (m, 2H, Ph 2,6-H), 8.42 (s, 1H, 5-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 0.32 (TMS), 18.7 ($CH(CH_3)_2$), 37.7 ($CH(CH_3)_2$), 96.1 ($C_4H_9C{\equiv}C$), 100.9 ($C_4H_9C{\equiv}C$), 119.9 (Ph C-2,6), 126.2 (C-4), 128.2 (Ph C-4), 129.7 (Ph C-3,5), 130.5 (C-5), 135.3 (C-3), 139.0 (Ph C-1), 199.2 (C=O). ^{15}N NMR (71 MHz, $CDCl_3$): δ –158.9 (N-1), N-2 was not found. MS m/z (%): 311 ($[M+H]^+$, 100). HRMS (ESI) for $C_{18}H_{22}N_2ONa$ ($[M+Na]^+$) calcd 333.1394, found 333.1394.

4.1.9. General procedure for the cyclization of 3-ethynyl-1-phenyl-1*H*-pyrazole-4-carbaldehydes (17-24), ethanones (25-31) and propanones (32-34).

A solution of compound **17-34** (0.5 mmol) in dry ammonia and methanol (NH_3/MeOH 2 M, 8 mL) was heated at 120 °C for 15 h in a steel reactor. The solvent was evaporated and the crude was purified by flash chromatography (SiO_2, eluent: ethyl acetate/n-hexane, 1:4, v/v) to yield compounds **35-51**.

4.1.9.1. 2-Methyl-6-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (35). White solid, yield 79%, 83 mg, mp 159-160 °C. IR (ν_{max}, cm^{-1}): 3106, 3062, 3030 (CH_{arom}), 2997, 2943 (CH_{aliph}), 1615, 1471, 1367, 1240, 1153 (C=C, C–N), 926, 858, 831, 755, 688, 677 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 4.27 (s, 3H, CH_3), 7.38-7.40 (m, 1H, Ph 4-H), 7.47-7.50 (m, 2H, Ph 3,5-H), 7.93 (s, 1H, 7-H), 8.05-8.09 (m, 2H, Ph 2,6-H), 8.08 (s, 1H, 3-H), 9.28 (s, 1H. 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 40.9 (CH_3), 107.0 (C-7), 119.4 (C-3a), 125.1 (C-3), 127.2 (Ph C-2,6), 128.4 (Ph C-4), 128.8 (Ph C-3,5), 140.3 (Ph C-1), 146.3 (C-4), 151.1 (C-6), 151.6 (C-7a). ^{15}N NMR (71 MHz, $CDCl_3$): δ –160.6 (N-2), –97.6 (N-1), –87.7 (N-5). MS m/z (%): 210 ($[M+H]^+$, 100). HRMS (ESI) for $C_{13}H_{12}N_3$ ($[M+H]^+$) calcd 210.1027, found 210.1026.

4.1.9.2. 2-Phenyl-6-(thiophen-3-yl)-2*H*-pyrazolo[4,3-*c*]pyridine (37). White solid, yield 94%, 130 mg, mp 129-130 °C. IR (ν_{max}, cm^{-1}): 3132, 3113, 3064, 3041 (CH_{arom}), 2920, 2851 (CH_{aliph}), 1506, 1362, 1254, 1202, 1039 (C=C, C–N), 862, 803, 760, 750, 687 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 7.42-7.43 (m, 1H, Th 4-H), 7.47-7.49 (m, 1H, Ph 4-H), 7.56-7.59 (m, 2H, Ph 3,5-H), 7.70-7.71 m, 1H, Th 5-H), 7.91 (s, 1H, 7-H),

7.92-7.94 (m, 2H, Ph 2,6-H), 8.00-8.01 (m, 1H, Th 2-H), 8.58 (d, ^4J(3-H,4-H) = 0.8 Hz, 1H, 3-H), 9.28 (d, ^4J(4-H,3-H) = 1.2 Hz 1H, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 106.4 (C-7), 119.8 (C-3a), 121.4 (Ph C-2,6), 122.1 (C-3), 123.3 (Th C-2), 126.2 (Th C-5), 126.4 (Th C-4), 129.0 (Ph C-4), 130.0 (Ph C-3,5), 140.1 (Ph C-1), 142.7 (Th C-3), 147.4 (C-4), 147.6 (C-6), 151.7 (C-7a). ^{15}N NMR (71 MHz, $CDCl_3$): δ −145.1 (N-2), −99.7 (N-1). MS m/z (%): 278 ($[M+H]^+$, 100). HRMS (ESI) for $C_{16}H_{12}N_3S$ ($[M+H]^+$) calcd 278.0746, found 278.0747.

4.1.9.3. 6-Cyclopropyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (38). White solid, yield 98%, 115 mg, mp 143-144 °C. IR (ν_{max}, cm^{-1}): 3116, 3090, 3036, 3006 (CH_{arom}), 2923, 2850 (CH_{aliph}), 1597, 1497, 1377, 1318, 1201, 1053, 1038 (C=C, C–N), 769, 761, 691 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.99-1.01 (m, 2H, CH_2CHCH_2), 1.05-1.07 (m, 2H, CH_2CHCH_2), 2.14-2.18 (m, 2H, CH_2CHCH_2), 7.40 (s, 1H, 7-H), 7.42-7.45 (m, 1H, Ph 4-H), 7.52-7.54 (m, 2H, Ph 3,5-H), 7.87-7.89 (m, 2H, Ph 2,6-H), 8.52 (s, 1H, 3-H), 9.12 (s, 1H, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 8.9 (CH_2CHCH_2), 17.5 (CH_2CHCH_2), 106.6 (C-7), 119.3 (C-3a), 121.4 (Ph C-2,6), 122.0 (C-3), 128.8 (Ph C-4), 129.8 (Ph C-3,5), 140.1 (Ph C-1), 146.9 (C-4), 151.6 (C-7a), 155.7 (C-6). ^{15}N NMR (71 MHz, $CDCl_3$): δ −145.3 (N-2), −91.0 (N-5), N-1 was not found. MS m/z (%): 236 ([M+H]+, 100). HRMS (ESI) for $C_{15}H_{14}N_3$ ($[M+H]^+$) calcd 236.1182 , found 236.1184.

4.1.9.4. 6-Butyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (39). Brown amorphous solid, yield 84%, 105 mg. IR (ν_{max}, cm^{-1}): 3138, 3013 (CH_{arom}), 2949, 2930, 2867 (CH_{aliph}), 1507, 1468, 1372, 1320, 1208, 1053, 1037 (C=C, C–N), 763, 751, 689 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.95-0.97 (m, 3H, $C_3H_6CH_3$), 1.40-1.45 (m, 2H, ($C_2H_4CH_2CH_3$), 1.76-1.80 (m, 2H, $CH_2CH_2C_2H_5$), 2.88-2.90 (m, 2H, $CH_2C_3H_7$), 7.40 (s, 1H, 7-H), 7.43-7.45 (m, 1H, Ph 4-H), 7.53-7.55 (m, 2H, Ph 3,5-H), 7.88-7.89 (m, 2H, Ph 2,6-H), 8.52 (s, 1H, 3-H), 9.18 (s, 1H, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 14.1 ($C_3H_6CH_3$), 22.6 ($C_2H_4CH_2CH_3$), 32.0 ($CH_2CH_2C_2H_5$), 38.3 ($CH_2(C_3H_7)$), 108.3 (C-7), 119.2 (C-3a), 121.4 (Ph C-2,6), 121.8 (C-3), 128.8 (Ph C-4), 129.9 (Ph C-3,5), 140.2 (Ph C-1), 147.0 (C-4), 151.7 (C-7a), 155.7 (C-6). ^{15}N NMR (71 MHz, $CDCl_3$): −145.5 (N-2), −78.9 (N-5), N-1 was not found. MS m/z (%): 252 ($[M+H]^+$, 100). HRMS (ESI) for $C_{16}H_{18}N_3$ ($[M+H]^+$) calcd 252.1495 , found 252.1497.

4.1.9.5. 6-Pentyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (40). Brown amorphous solid, yield 89%, 118 mg. IR (ν_{max}, cm^{-1}): 3122, 3063, 3040 (CH_{arom}), 2949, 2925, 2856 (CH_{aliph}), 1466,

1324, 1206, 1037 (C=C, C–N), 862, 759, 750, 684 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.89-0.90 (m, 2H, $CH_2(CH_2)_3CH_3$), 1.37-1.39 (m, 4H, $(CH_2)_2(CH_2)_2CH_3$), 1.78-1.81 (m, 2H, CH_2CH_2 $(CH_2)_2CH_3$), 2.86-2.89 (m, 2H, $CH_2(CH_2)_3CH_3$), 7.40 (s, 1H, 7-H), 7.43-7.44 (m, 1H, Ph 4-H), 7.52-7.55 (m, 2H, Ph 3,5-H), 7.88-7.89 (m, 2H, Ph 2,6-H), 8.52 (s, 1H, 3-H), 9.18 (s, 1H, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 14.2 ($C_4H_8CH_3$), 22.7 ($C_3H_6CH_2CH_3$), 29.6 ($CH_2CH_2C_3H_7$), 31.7 ($C_2H_4CH_2C_2H_5$), 38.6 ($CH_2C_4H_{11}$), 108.3 (C-7), 119.1 (C-3a), 121.4 (Ph C-2,6), 121.8 (C-3), 128.8 (Ph C-4), 129.8 (Ph C-3,5), 140.2 (Ph C-1), 146.9 (C-6), 151.7 (C-4), 155.8 (C-7a). ^{15}N NMR (71 MHz, $CDCl_3$): δ −145.3 (N-2), −79.8 (N-5), N-1 was not found. MS m/z (%): 266 ($[M+H]^+$, 100). HRMS (ESI) for $C_{17}H_{20}N_3$ ($[M+H]^+$) calcd 266.1652 , found 266.1652.

4.1.9.6. 2-(2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridin-6-yl)ethanol (41). Brown solid, yield 86%, 103 mg, mp 215-216 °C. IR (ν_{max}, cm^{-1}): 3187 (OH), 3108, 3067, 3022 (CH_{arom}), 2961, 2935, 2916 (CH_{aliph}), 1503, 1325, 1227, 1213, 1056 (C=C, C–N), 765, 752, 677 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.11-3.12 (m, 2H, CH_2CH_2OH), 4.05-4.06 (m, 2H, CH_2CH_2OH),), 7.45-7.48 (m, 2H, H-7, Ph 4-H), 7.55-7.58 (m, 2H, Ph 3,5-H), 7.89-7.90 (m, 2H, Ph 2,6-H), 8.58 (s, 1H, 3-H), 9.16 (s, 1H, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 39.6 (CH_2CH_2OH) 62.7 (CH_2CH_2OH), 109.5 (C-7), 119.4 (C-3a), 121.5 (Ph C-2,6), 122.3 (C-3), 129.1 (Ph C-4), 129.9 (Ph C-3,5), 140.0 (Ph C-1), 151.3 (C-4), 153.6 (C-7a). ^{15}N NMR (71 MHz, $CDCl_3$): δ −144.3 (N-2), −100.5 (N-1), −88.3 (N-5). MS m/z (%): 240 ($[M+H]^+$, 100). HRMS (ESI) for $C_{14}H_{14}N_3O$ ($[M+H]^+$) calcd 240.1131, found 240.1131.

4.1.9.7. 2-Phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (42). White solid, yield 95%, 193 mg, mp 132 °C (lit mp 132-133 °C [35]). IR (ν_{max}, cm^{-1}): 3129, 3094, 3064 (CH_{arom}), 1614, 1490, 1466, 1365, 1349, 1315, 1210, 1178 (C=C, C–N), 916, 827, 760, 750, 684, 587, 508, 431 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 7.45-7.48 (m, 1H, Ph 4-H), 7.54-7.56 (m, 2H, Ph 3,5-H), 7.62-7.64 (m, 1H, 7-H), 7.90-7.91 (m, 2H, Ph 2,6-H), 8.31-8.32 (m, 1H, 6-H), 8.63 (s, 1H, 3-H), 9.27 (4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 111.8 (C-7), 120.5 (C-3a), 121.6 (Ph C-2,6), 122.4 (C-3), 129.1 (Ph C-4), 129.9 (Ph C-3,5), 140.0 (Ph C-1), 142.1 (C-6), 147.5 (C-4), 150.4 (C-7a). ^{15}N NMR (71 MHz, $CDCl_3$): δ −147.4 (N-2), –99.6 (N-1), –89.8 (N-5). MS m/z (%): 196 ($[M+H]^+$, 100). HRMS (ESI) for $C_{12}H_{10}N_3$ ($[M+H]^+$) calcd 196.0869, found 196.0869.

4.1.9.8. 4-Methyl-2-phenyl-6-(thiophen-3-yl)-2*H*-pyrazolo[4,3-*c*]pyridine (44). White solid, yield 81%, 118 mg, mp 92-93 °C. IR (ν_{max}, cm^{-1}): 3129, 3104, 3072 (CH_{arom}), 2982, 2948, 2911 (CH_{aliph}), 1546, 1507, 1374, 1202, 1045 (C=C, C–N), 849, 796, 763, 744, 690 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 2.86 (s, 3H, CH_3), 7.39-7.40 (m, 1H, Th 4-H), 7.43-7.45 (m, 1H, Ph 4-H), 7.53-7.55 (m, 2H, Ph 3,5-H), 7.67-7.68 (m, 1H, Th 5-H), 7.72 (s, 1H, 7-H), 7.90-7.91 (m, 2H, Ph 2,6-H), 8.01-8.02 (m, 1H, Th 2-H), 8.49 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 23.3 (CH_3), 104.3 (C-7), 119.7 (C-3a), 121.2 (Ph C-2,6), 121.9 (C-3), 123.2 (Th C-2), 126.2 (Th C-4,5), 128.7 (Ph C-4), 129.8 (Ph C-3,5), 140.1 (Ph C-1), 142.8 (Th C-3), 147.3 (C-4), 152.0 (C-7a), 156.2 (C-6). ^{15}N NMR (71 MHz, $CDCl_3$): δ −147.4 (N-2), –99.6 (N-1), –89.8 (N-5). MS m/z (%): 292 ($[M+H]^+$, 100). HRMS (ESI) for $C_{17}H_{14}N_3S$ ($[M+H]^+$) calcd 292.0903, found 292.0903.

4.1.9.9. 6-Cyclopropyl-4-methyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (45). White solid, yield 80%, 99 mg, mp 85-86 °C. IR (ν_{max}, cm^{-1}): 3129, 3078, 3044 (CH_{arom}), 2996, 2911, 2850 (CH_{aliph}), 1500, 1401, 1300, 1202, 1071, 1041 (C=C, C–N), 821, 760, 750, 685, 538 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.95-0.98 (m, 2H, CH_2CHCH_2), 0.99-1.01 (m, 2H, CH_2CHCH_2), 2.14-2.16 (m, 2H, CH_2CHCH_2), 2.75 (s, 3H, CH_3), 7.16 (s, 1H, 7-H), 7.40-7.42 (m, 1H, Ph 4-H), 7.50-7.53 (m, 2H, Ph 3,5-H), 7.86-7.87 (m, 2H, Ph 2,6-H), 8.44 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 8.7 (CH_2CHCH_2), 17.6 (CH_2CHCH_2), 23.0 (CH_3), 103.6 (C-7), 119.4 (C-3a), 121.2 (Ph C-2,6), 121.7 (C-3), 128.5 (Ph C-4), 129.8 (Ph C-3,5), 140.2 (Ph C-1), 152.0 (C-7a), 155.6 (C-4), 155.9 (C-6). ^{15}N NMR (71 MHz, $CDCl_3$): δ −147.9 (N-2), −90.4 (N-5), N-1 was not found. MS m/z (%): 250 ($[M+H]^+$, 100). HRMS (ESI) for $C_{16}H_{16}N_3$ ($[M+H]^+$) calcd 250.1339, found 250.1337.

4.1.9.10. 6-Butyl-4-methyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (46). Brown liquid, yield 88%, 116 mg. IR (ν_{max}, cm^{-1}): 3070 (CH_{arom}), 2955, 2928, 2870 (CH_{aliph}), 1618, 1597, 1544, 1510, 1374, 1165, 1043 (C=C, C–N), 758, 688 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.94-0.97 (m, 3H, $C_3H_6CH_3$), 1.40-1.45 (m, 2H, $(C_2H_4CH_2CH_3$), 1.71-1.79 (m, 2H, $CH_2CH_2C_2H_5$), 2.81 (s, 3H, CH_3) 2.81-2.86 (m, 2H, $CH_2C_3H_7$), 7.24 (s, 1H, 7-H), 7.42-7.44 (m, 1H, H-4), 7.52-7.54 (m, 2H, Ph 3,5-H), 7.88-7.89 (m, 2H, Ph 2,6-H), 8.49 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 14.2 ($C_3H_6CH_3$), 22.6 ($C_2H_4CH_2CH_3$), 23.1 (CH_3), 32.1 ($CH_2CH_2C_2H_5$), 38.3 ($CH_2(C_3H_7)$, 106.0 (C-7), 119.2 (C-3a), 121.3 (Ph C-2,6), 121.8 (C-3), 128.6 (Ph C-4), 129.8 (Ph C-3,5), 140.2 (Ph C-1), 152.0 (C-7a), 155.5 (C-4), 155.6 (C-6). ^{15}N

NMR (71 MHz, $CDCl_3$): δ −147.6 (N-2), −85.2 (N-5), N-1 was not found. MS m/z (%): 266 ($[M+H]^+$, 100). HRMS (ESI) for $C_{17}H_{20}N_3$ ($[M+H]^+$) calcd 266.1652, found 266.1654.

4.1.9.11. 4-Methyl-6-pentyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (47). Brown liquid, yield 88%, 123 mg. IR (ν_{max}, cm^{-1}): 3100, 3071 (CH_{arom}), 2954, 2928, 2870, 2857 (CH_{aliph}), 1619, 1597, 1544, 1510, 1374, 1309, 1043 (C=C, C–N), 758, 688 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.82-0.91 (m, 2H, $C_4H_{11}CH_3$), 1.35-1.41 (m, 4H, $C_2H_4(CH_2)_2CH_3$), 1.76-1.80 (m, 2H, $CH_2CH_2C_3H_7$), 2.82 (CH_3), 2.84-2.86 (m, 2H, $CH_2C_4H_9$), 7.24 (s, 1H, H-7), 7.42-7.45 (m, 1H, Ph 4-H), 7.53-7.55 (m, 2H, Ph 3,5-H), 7.88-7.89 (m, 2H, Ph 2,6-H), 8.50 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 14.2 ($C_4H_8CH_3$), 22.8 ($C_3H_6CH_2CH_3$), 23.0 (CH_3), 29.6 ($CH_2CH_2C_3H_7$), 31.7 ($C_2H_4CH_2C_2H_5$), 38.4 ($CH_2C_4H_{11}$), 106.1 (C-7), 119.2 (C-3a), 121.3 (Ph C-2,6), 121.9 (C-3), 128.7 (Ph C-4), 129.8 (Ph C-3,5), 140.2 (Ph C-1), 152.0 (C-7a), 155.4 (C-4), 155.6 (C-6). ^{15}N NMR (71 MHz, $CDCl_3$): δ −148.9 (N-2), −85.0 (N-5), N-1 was not found. MS m/z (%): 280 ($[M+H]^+$, 100). HRMS (ESI) for $C_{18}H_{22}N_3$ ($[M+H]^+$) calcd 280.1808, found 280.1808.

4.1.9.12. 2-(4-Methyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridin-6-yl)ethanol (48). White solid, yield 86%, 109 mg, mp 126-127 °C. IR (ν_{max}, cm^{-1}): 3227 (OH), 3149, 3072, 3018 (CH_{arom}), 2945, 2919, 2870 (CH_{aliph}), 1622, 1501, 1389, 1374, 1306, 1212, 1164, 1053 (C=C, C–N), 844, 756, 741, 682, 571 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 2.74 (s, 3H, CH_3), 3.03 (t, *J*=5.5 Hz, 2H, CH_2CH_2OH), 4.00-4.02 (m, 2H, CH_2CH_2OH), 7.22 (s, 1H, 7-H), 7.40-7.42 (m, 2H, H-7, Ph 4-H), 7.49-7.51 (m, 2H, Ph 3,5-H), 7.84-7.85 (m, 2H, Ph 2,6-H), 8.48 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 23.0 (CH_3), 39.4 (CH_2CH_2OH) 62.7 (CH_2CH_2OH), 107.0 (C-7), 119.2 (C-3a), 121.2 (Ph C-2,6), 122.0 (C-3), 128.7 (Ph C-4), 129.8 (Ph C-3,5), 140.0 (Ph C-1), 151.5 (C-7a), 153.4 (C-6), 155.7 (C-4). ^{15}N NMR (71 MHz, $CDCl_3$): δ −146.8 (N-2), −92.3 (N-5), N-1 was not found. MS m/z (%): 254 ($[M+H]^+$, 100). HRMS (ESI) for $C_{15}H_{16}N_3O$ ($[M+H]^+$) calcd 254.1288, found 254.1287.

4.1.9.13. 4-Methyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (49). Brown amorphous solid, yield 88%, 92 mg. IR (ν_{max}, cm^{-1}): 3066 (CH_{arom}), 2995 (CH_{aliph}), 1596, 1498, 1416, 1370, 1345, 1237, 1190, 1031 (C=C, C–N), 807, 756, 746, 687, 638, 529 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 2.85 (s, 3H, CH_3), 7.46-7.48 (m, 2H, 7-H, Ph 4-H), 7.55-7.58 (m, 2H, Ph 3,5-H), 7.91-7.93 (m, 2H, Ph 2,6-H), 8.20 (d, ^3J(6-H,7-H) = 6.4 Hz, 1H, 6-H), 8.58 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 23.0 (CH_3) 109.5 (C-7), 120.6 (C-3a), 121.5 (Ph C-

2,6), 122.0 (C-3), 128.9 (Ph C-4), 129.9 (Ph C-3,5), 140.1 (Ph C-1), 142.1 (C-6), 150.9 (C-4), 156.4 (C-7a). ^{15}N NMR (71 MHz, $CDCl_3$): δ −146.9 (N-2), −98.48 (N-1), −91.0 (N-5). MS m/z (%): 210 ($[M+H]^+$, 100). HRMS (ESI) for $C_{12}H_{13}N_3$ ($[M+H]^+$) calcd 210.1026, found 210.1026.

4.1.9.14. 4-Isopropyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine (50). White solid, yield 92%, 143 mg, mp 97-98 °C. IR (ν_{max}, cm^{-1}): 3126, 3064, 3023 (CH_{arom}), 2960, 2924, 2898 (CH_{aliph}), 1600, 1510, 1466, 1399, 1314, 1277, 1203, 1046 (C=C, C–N), 767, 760, 756, 696, 684, 635 (CH=CH of monosubstituted benzenes). ^{1}H NMR (700 MHz, $CDCl_3$): δ 1.54 (d, *J* = 7.0 Hz, 6H, $CH(CH_3)_2$), 3.52-3.56 (m, 1H, $CH(CH_3)_2$), 7.38-7.40 (m, 1H, CPh 4-H), 7.44-7.47 (m, 1H, NPh 4-H), 7.48-7.52 (m, 2H, CPh 3,5-H), 7.55-7.58 (m, 2H, NPh 3,5-H), 7.89 (s, 1H, 7-H), 7.93-7.96 (m, 2H, NPh 2,6-H), 8.18-8.19 (m, 2H, CPh 2,6-H), 8.57 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 21.9 ($CH(CH_3)_2$), 35.8 ($CH(CH_3)_2$), 104.5 (C-7), 118.0 (C-3), 121.2 (NPh C-2,6), 127.0 (CPh C-2,6), 128.2 (CPh C-4), 128.56 (CPh C-3,5), 128.62 (NPh C-4), 129.7 (NPh C-3,5), 140.1 (CPh C-1), 140.4 (NPh C-1), 150.5 (C-6), 152.8 (C-7a), 164.1 (C-4). ^{15}N NMR (71 MHz, $CDCl_3$): δ −148.9 (N-2), −86.5 (N-1), −90.8 (N-5). MS m/z (%): 314 ($[M+H]^+$, 100). HRMS (ESI) for $C_{21}H_{20}N_3$ ($[M+H]^+$) calcd 314.1652, found 314.1652.

4.1.9.15. 6-Butyl-4-isopropyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (51). Brown liquid, yield 84%, 123 mg. IR (ν_{max}, cm^{-1}): 3111, 3065, 3047 (CH_{arom}), 2959, 2929 (CH_{aliph}), 1615, 1598, 1541, 1510, 1467, 1376, 1306, 1275, 1211, 1196, 1161, 1044 (C=C, C–N), 848, 757, 688 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 0.95-0.97 (m, 3H, $C_3H_6CH_3$), 1.40-1.44 (m, 2H, ($C_2H_4CH_2CH_3$), 1.44-1.47 (m, 6H, $CH(CH_3)_2$), 1.75-1.80 (m, 2H, $CH_2CH_2C_2H_5$), 2.85-2.90 (m, 2H, $CH_2C_3H_7$), 3.41-3.45 (s, 1H, $CH(CH_3)_2$), 7.23 (s, 1H, 7-H), 7.42-7.46 (m, 1H, H-7), 7.53-7.54 (m, 2H, Ph 3,5-H), 7-89-7.90 (m, 2H, Ph 2,6-H), 8.53 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 14.2 ($C_3H_6CH_3$), 22.1 ($CH(CH_3)_2$), 22.6 ($C_2H_4CH_2CH_3$), 31.8 ($CH_2CH_2C_2H_5$), 36.3 ($CH(CH_3)_2$), 38.3 ($CH_2(C_3H_7)$, 105.8 (C-7), 116.9 (C-3a), 121.3 (C-3), 121.4 (Ph C-2,6), 128.5 (Ph C-4), 129.8 (Ph C-3,5), 140.3 (Ph C-1), 152.8 (C-7a), 155.4 (C-6), 164.1 (C-4). ^{15}N NMR (71 MHz, $CDCl_3$): δ −147.4 (N-2), −99.6 (N-1), −90.8 (N-5). MS m/z (%): 294 ($[M+H]^+$, 100). HRMS (ESI) for $C_{19}H_{24}N_3$ ($[M+H]^+$) calcd 294.1965 , found 294.1965.

4.1.9.16. 4-Isopropyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine (52). Brown liquid, yield 92%, 109 mg. IR (ν_{max}, cm^{-1}): 3061 (CH_{arom}), 2966, 2927, 2869 (CH_{alif}), 1609, 1510, 1498, 1369, 1276, 1238, 1193, 1026 (C=C, C–N), 806, 757, 688 (CH=CH of monosubstituted benzene). ^{1}H NMR (700 MHz, $CDCl_3$): δ 1.47 (d, *J* = 7.1 Hz, 6H, $CH(CH_3)_2$), 3.46-3.50 (m, 1H, $CH(CH_3)_2$), 7.44-

7.47 (m, 2H, 7-H, Ph 4-H,), 7.54-7.56 (m, 2H, Ph 3,5-H), 7.90-7.92 (m, 2H, Ph 2,6-H), 8.25 (d, ^{3}J(6-H,7-H) = 6.4 Hz, 1H, 6-H), 8.60 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 21.8 ($CH(CH_3)_2$, 35.4 ($CH(CH_3)_2$), 109.4 (C-7), 118.7 (C-3a), 121.4 (Ph C-2,6), 121.5 (C-3), 128.7 (Ph C-4), 129.7 (Ph C-3,5), 140.0 (C-6), 142.1 (Ph C-1), 151.2 (C-7a), 165.0 (C-4). ^{15}N NMR (71 MHz, $CDCl_3$): δ −147.4 (N-2), −100.6 (N-1), −95.3 (N-5). MS m/z (%): 238 ($[M+H]^+$, 100). HRMS (ESI) for $C_{15}H_{16}N_3$ ($[M+H]^+$) calcd 238.1339 , found 238.1339.

4.2. Cancer cell lines and cytotoxicity assay

Human cancer cell lines were obtained from the American Type Culture Collection and were cultivated according to the provider's instructions. In brief, MCF-7 and K562 cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 °C in 5% CO_2. For the cytotoxicity assays, cells were treated in triplicate with six different doses of each compound for 72 hours. After treatment, Calcein AM solution was added for 1 hour, and the fluorescence from live cells was measured at 485 nm/538 nm (excitation/emission) using a Fluoroskan Ascent microplate reader (Labsystems). The GI_{50} value, the drug concentration lethal to 50% of the cells, was calculated from the dose-response curves that resulted from the assays.

4.3. Flow cytometry

Asynchronous cells were seeded into a 96 well plate and then, after a preincubation period, treated with the tested compounds for 24 hours at a single dose of 10 µM. MCF-7 cells were first washed with PBS, trypsinized, and finally treated with a solution of trypsin inhibitor (0.1 %). After incubation, 5× staining solution (17 mM trisodium citrate dihydrate, 0.5% IGEPAL® CA-630, 7.5 mM spermine tetrahydrochloride, 2.5 mM Tris; pH 7.6 containing 50 µg/mL propidium iodide) was added. K-562 cells were stained directly with the 5x staining solution (i.e. without trypsinization). The cells' DNA content was analyzed by flow cytometry using a 488 nm laser (BD FACS Verse with software BD FACSuite™, version 1.0.6.). Cell cycle distribution was analyzed using ModFit LT (Verity Software House, version 4.1.7).

For quantification of histone H3 phosphorylation, the cells were harvested by trypsinization, washed in PBS, fixed with ice-cold 90% methanol, incubated on ice for 30 min and washed with PBS/BSA containing 0.5% Tween-20. Then, the cells were incubated with the primary antibody

raised against histone H3 phosphorylated at Ser10 (Millipore) for 1 hour at room temperature, washed with PBS containing 1% BSA and incubated with the secondary antibody (goat-anti-rabbit-Alexa Fluor 488, Invitrogen) for 1 hour in the dark. After washing with PBS/BSA, each sample was incubated with propidium iodide (final concentration 10 µg/mL) and RNAse A (final concentration 200 µg/mL) for 30 minutes at room temperature in the dark. The cells were then analyzed by flow cytometry using a 488 nm laser (BD FACS Verse with software BD FACSuite^{TM}, version 1.0.6.).

4.4. Caspase-3/7 assay

Cellular caspase-3/7 activity was measured according to previously published procedure [39]. K562 cells were cultivated in a 96-well plate overnight. Next day, the cells were treated with increasing concentrations of compound **5** for next 24 hours. After incubation, 3x caspase-3/7 assay buffer (150 mM HEPES pH 7.4, 450 mM NaCl, 150 mM KCl, 30 mM $MgCl_2$, 1.2 mM EGTA, 1.5% Nonidet P40, 0.3% CHAPS, 30% sucrose, 30 mM DTT, 3 mM PMSF) containing 150 µM peptide substrate Ac-DEVD-AMC (Enzo Life Sciences) was added and after 2 hours incubation, the caspase-3/7 activity was measured using Fluoroskan Ascent microplate reader (Labsystems) at 346 nm/442 nm (excitation/emission). The activity was normalized to an untreated control.

4.5. Immunoblotting

Immunoblotting was performed as described earlier [40]. In brief, cellular lysates were prepared by harvesting cells in Laemmli sample buffer. Proteins were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. After blocking, the membranes were incubated with specific primary antibodies overnight, washed and then incubated with peroxidase-conjugated secondary antibodies. Finally, peroxidase activity was detected with ECL+ reagents (AP Biotech) using a CCD camera LAS-4000 (Fujifilm). Specific antibodies were purchased from Santa Cruz Biotechnology (PARP, β-actin), Cell Signaling (Ser-139 phosphorylated H2AX, Bcl-2) and Sigma Aldrich (peroxidase-labeled secondary antibodies).

Author contributions

E.A., W.H., V.K., and A.Š. designed the research; V.M.[1], E.A., E.Ř., R.J., and V.M.[3] performed the research; V.M.[1], E.A., E.Ř., R.J., V.M.[3], A.Ž., W.H,. V.K., and A.Š. analyzed the data; V.M.[1], E.A., A.Ž., and V.K. wrote the manuscript; all authors approved the final version of the manuscript.

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